Peroxisomal oxidation of erucic acid suppresses mitochondrial fatty acid oxidation by stimulating malonyl-CoA formation in the rat liver

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Feeding of rapeseed (canola) oil with a high erucic acid concentration is known to cause hepatic steatosis in animals. Mitochondrial fatty acid oxidation plays a central role in liver lipid homeostasis, so it is possible that hepatic metabolism of erucic acid might decrease mitochondrial fatty acid oxidation. However, the precise mechanistic relationship between erucic acid levels and mitochondrial fatty acid oxidation is unclear. Using male Sprague-Dawley rats, along with biochemical and molecular biology approaches, we report here that peroxisomal β-oxidation of erucic acid stimulates malonyl-CoA formation in the liver and thereby suppresses mitochondrial fatty acid oxidation. Excessive hepatic uptake and peroxisomal β-oxidation of erucic acid resulted in appreciable peroxisomal release of free acetate, which was then used in the synthesis of cytosolic acetyl-CoA. Peroxisomal metabolism of erucic acid also remarkably increased the cytosolic NADH/NAD⁺ ratio, suppressed sirtuin 1 (SIRT1) activity, and thereby activated acetyl-CoA carboxylase, which stimulated malonyl-CoA biosynthesis from acetyl-CoA. Chronic feeding of a diet including high-erucic-acid rapeseed oil diminished mitochondrial fatty acid oxidation and caused hepatic steatosis and insulin resistance in the rats. Of note, administration of a specific peroxisomal β-oxidation inhibitor attenuated these effects. Our findings establish a cross-talk between peroxisomal and mitochondrial fatty acid oxidation. They suggest that peroxisomal oxidation of long-chain fatty acids suppresses mitochondrial fatty acid oxidation by stimulating malonyl-CoA formation, which might play a role in fatty acid–induced hepatic steatosis and related metabolic disorders.

It is well-known that high-erucic-acid rapeseed oil feeding develops transient cardiac lipidosis in animals as well as in humans, and the imbalance between the input and oxidation of erucic acid was proposed to be a critical cause for the acute lipid deposition in heart (1–3). High-erucic-acid rapeseed oil feeding also caused lipid deposition in liver; however, the effect of erucic acid on hepatic steatosis was progressive and irreversible (4, 5); therefore, the mechanism that led to lipid deposition in liver was distinct from that in heart. As mitochondrial fatty acid oxidation plays a central role in liver fatty acid metabolism, it is rational to assume that metabolism of erucic acid might negatively regulate mitochondrial fatty acid oxidation and lead to hepatic steatosis. However, the precise mechanism linking erucic acid and liver mitochondrial fatty acid oxidation is not clear.

To explore the potential mechanism, we focused on peroxisomal β-oxidation system, a fatty acid oxidation (FAO) system that acted exclusively on very long-chain and branched-chain fatty acids (6). As a very long-chain fatty acid, erucic acid was reported to be preferentially metabolized by the peroxisomal β-oxidation system (7, 8). Interestingly, PPARα was activated, and peroxisomal fatty acid oxidation is extensively induced in animals fed a high-erucic-acid rapeseed oil diet (9–11), which led to accelerated turnover of erucic acid as well as long-chain fatty acids in peroxisomes. It has been suggested that the peroxisomal β-oxidation system and mitochondrial fatty acid metabolism system are mutually competitive; inhibition of peroxisomal β-oxidation stimulated mitochondrial β-oxidation in a previous report (12). We therefore hypothesized that excessive oxidation of erucic acid by peroxisomes might negatively regulate mitochondrial fatty acid oxidation and lead to lipid deposition in liver. This study investigated the effect of peroxisomal oxidation of erucic acid on mitochondrial fatty acid oxidation as well as the potential mechanism by which metabolism of erucic acid leads to hepatic steatosis in animals.

Results

Erucic acid was mainly metabolized by the peroxisomal β-oxidation system

To determine the cellular compartmentation of erucic acid oxidation, the kinetic parameters of key enzymes involved in mitochondrial or peroxisomal FAO were determined (Fig. 1A). For carnitine palmitoyltransferase-1 (CPT1), the enzyme responsible for the transport of long-chain fatty acids into mitochondria, the Vmax value for C22:1-CoA (erucyl-CoA) is nearly 1 order of magnitude lower than that for C16-CoA (palmitoyl-CoA), whereas its Km value was 1 order of magnitude higher, which was in accordance with previous reports that CPT1 or CPT2 showed no obvious activity toward very long-chain fatty acid (>C22) (13, 14). For acyl-CoA dehydrogenases, which catalyze the first step of mitochondrial β-oxidation, short-chain (SCAD) and medium-chain (MCAD) acyl-CoA dehydrogenases are completely inactive with C22:1-CoA. As for long-chain acyl-CoA dehydrogenase (LCAD), a significantly lower Vmax value was observed along with a higher Km value for C22:1-CoA as compared with C16-CoA, indicating that C22:1-CoA was not preferentially oxidized by mitochondria. Acyl-

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CoA oxidase-1 (ACOX1), the rate-limiting enzyme of peroxisomal \( \beta \)-oxidation, showed a relatively high activity for C22:1-CoA with \( V_{\text{max}} \) and \( K_m \) values at the same order of magnitude compared with C16-CoA. Moreover, the capacities of subcellular fractions for erucic acid oxidation were determined using isolated peroxisomes or mitochondria (Fig. 1A). This system showed relatively high activity for C22:1-CoA, which was strongly enhanced by treatment with clofibrate (CFB), a classical PPAR\( \alpha \) activator (15), and suppressed by the addition of 10,12-tricosadiynoic acid (TDYA)-CoA, a specific inhibitor for ACOX1 (12). Furthermore, the metabolism of erucic acid by liver homogenate led to dose-dependent generation of hydrogen peroxide, a byproduct of peroxisomal \( \beta \)-oxidation, as inhibited by TDYA-CoA (Fig. 1C). All of the results supported the hypothesis that erucic acids are preferentially metabolized by peroxisomal FAO system, which was in agreement with previous reports (7, 8).

### Peroxisomal \( \beta \)-oxidation was significantly enhanced in livers of the rats fed high-erucic-acid rapeseed oil (HRO)

To selectively increase the liver oxidation of erucic acid, CFB was used to strongly induce peroxisomal \( \beta \)-oxidation (15). TDYA, a specific inhibitor for peroxisomal \( \beta \)-oxidation, was applied to inhibit liver peroxisomal \( \beta \)-oxidation (12).

Activation of PPAR\( \alpha \) triggers downstream transcription of genes involved in peroxisomal \( \beta \)-oxidation (6, 16, 17). Expression of genes involved in peroxisomal \( \beta \)-oxidation were up-regulated by HRO feeding, suggesting that erucic acid was a potential endogenous ligand for PPAR\( \alpha \), and CFB treatment caused robust induction of peroxisomal \( \beta \)-oxidation in high-oleic-acid diet (HOO)- and HRO-fed rats (Fig. 2A). Peroxisomal FAO was significantly induced in livers of the rats fed the HRO diet, and administration of CFB strongly stimulated peroxisomal \( \beta \)-oxidation in HOO- and HRO-fed rats, whereas peroxisomal \( \beta \)-oxidation was suppressed by treatment with TDYA (Fig. 2B). HRO feeding increased hepatic hydrogen peroxide production level (by 357% \( \pm \) 59% versus the HOO and LRO groups, respectively), CFB treatment further elevated hepatic hydrogen peroxide generation in the HOO-fed (by 386% \( \pm \) 27% versus the HOO group) and HRO-fed groups (by 123% \( \pm \) 67% versus the HRO group), and TDYA treatment reduced hydrogen peroxide generation in the liver (Fig. 2C). These results suggested that the presence of erucic acid in the diet activated PPAR\( \alpha \) and accelerated liver erucic acid oxidation, as strongly induced by CFB.

### Peroxisomal \( \beta \)-oxidation of erucic acid led to hepatic lipid deposition

Liver long-chain acyl-CoAs increased significantly in HRO-fed rats (by 133% and 90% versus the HOO and LRO groups, respectively), and CFB treatment further elevated long-chain acyl-CoAs in HRO-fed rats, as reduced by the treatment with TDYA (Fig. 3A). HRO feeding led to triacylglyceride (TAG) accumulation in rat liver (by 109 and 95% versus the HOO and LRO groups, respectively), as further elevated after treatment of CFB (increased by 105% versus the HOO group) and reduced by TDYA (Fig. 3B); CFB treatment also caused significant elevation in liver TAG in HOO-fed rats (by 70% versus the HOO group). HRO feeding increased the density of fat droplets in liver sections (23.5 \( \pm \) 4.4/1000 \( \mu \)m\(^2\) versus 3.4 \( \pm \) 0.6/1000 \( \mu \)m\(^2\)) and 3.9 \( \pm \) 0.7/1000 \( \mu \)m\(^2\) in the HOO and LRO groups, respectively). Lipid deposition in the HOO+CFB group was even more serious, where the size of lipid droplets was significantly increased (with average diameters of 5.06 \( \pm \) 1.41 \( \mu \)m) compared with the LRO and HRO groups (with average diameters of 1.54 \( \pm \) 0.12 and 3.49 \( \pm \) 0.28 \( \mu \)m, respectively). TDYA treatment significantly reduced lipid deposition in HRO-fed rats (Fig. 3C). HRO feeding also led to increased liver index (by 29 and 26% versus the HOO and LRO groups, respectively), and CFB treatment further elevated liver index in HOO- as well as HRO-fed rats, as decreased after treatment of TDYA (Fig. 3D). Plasma TAG was significantly higher in HRO-fed rats compared with the HOO and LRO groups (by 68 and 46%, respectively), as further elevated by the treatment of CFB and lowered by TDYA (Fig. 3E). Daily body weight gain increased by 17% in the HRO group compared with the LRO group, and TDYA significantly decreased body weight gain (by 18% versus the HRO group) (Fig. 3F). HRO feeding led to a significant increase in homeostasis model assessment of insulin resistance (HOMA-IR) index (by 118% versus LRO), and administration of TDYA to the rats fed HRO caused a significant decrease in the HOMA-IR index (Fig. 3G). Oral glucose intolerance was evident in HRO-fed rats, as shown by a significantly higher glucose curve compared with the
normal and LRO-fed rats, which was exacerbated after treatment of CFB and improved by TDYA (Fig. 3H). These results suggested that excessive oxidation of erucic acid in peroxisomes led to significant lipid deposition in the liver and insulin resistance in rats.

Peroxisomal β-oxidation of erucic acid suppressed mitochondrial β-oxidation by stimulating malonyl-CoA formation

Excessive peroxisomal β-oxidation of erucic acid led to lipid deposition in rat liver, suggesting a potential cross-talk between

Figure 2. HRO feeding enhanced liver peroxisomal β-oxidation. A, gene expression of the enzymes involved in peroxisomal β-oxidation of rat liver was up-regulated by HRO feeding, and CFB treatment strongly induced peroxisomal β-oxidation in HOO- and HRO-fed rats. B, liver peroxisomal β-oxidation activity was elevated in rats feeding HRO, as further enhanced by the treatment of CFB and inhibited by TDYA. C, HRO feeding increased hydrogen peroxide generation in rat liver, as further elevated by the treatment of CFB and reduced by TDYA. CFB treatment also significantly increased hydrogen peroxide generation in HOO-fed rats. Results are mean ± S.E. (error bars); *, p < 0.05 by t test between paired conditions.

Figure 3. HRO feeding led to hepatic steatosis and insulin resistance in rats. A, liver long-chain (LC) acyl-CoA was significantly higher in HRO-fed rats, as further elevated in the CFB-treated rats and reduced by TDYA. B, liver TAG was significantly higher in the HRO group, which was further increased by the treatment of CFB and reduced by TDYA. CFB treatment also led to a significant increase in liver TAG in HOO-fed rats. C, significant hepatic steatosis was observed in HRO and HOO + CFB groups. CFB treatment exacerbated and TDYA relieved hepatic steatosis in HRO-fed rats. Magnification: ×200. D, liver index was increased by HRO feeding, which was further elevated by CFB treatment, and decreased by TDYA. CFB treatment also significantly increased the liver index in HOO-fed rats. E, plasma TAG level was significantly higher in the rats fed HRO, as further elevated by CFB and reduced by TDYA. F, HRO treatment increased body weight gain, as further elevated by CFB and reduced by TDYA. G, HOMA-IR was significantly higher in HRO-fed rats, as further enhanced by the treatment of CFB and lowered by TDYA. H, oral glucose intolerance was evident in HRO-fed rats, as exacerbated by the treatment of CFB and improved by TDYA. Results are mean ± S.E. (error bars); *, p < 0.05 by t test between paired conditions.
mitochondria and peroxisome in which mitochondrial FAO might be suppressed because the mitochondrial FAO system plays a central role in liver lipid homeostasis. A previous report (12) also indicated that the peroxisomal and mitochondrial fatty acid metabolism systems are mutually competitive. Mitochondrial fatty acid oxidation in isolated hepatocytes was first measured by $^{14}$CO$_2$ formation from [1-$^{14}$C]palmitate, and the results suggested that the capacity of mitochondrial $\beta$-oxidation was significantly lowered in the liver of the HRO-fed rats (by 45 and 42% versus the HOO and LRO groups, respectively), which was recovered by TDYA (Fig. 4A). Mitochondrial $\beta$-oxidation was also significantly lower in the HOO+CFB group compared with the HOO group (decreased by 44%). The rate of ketogenesis using palmitate as a substrate was significantly lower in isolated hepatocytes from the HRO-fed rats (by 45 and 42% versus the HOO and LRO groups, respectively), as further suppressed by CFB (by 37% versus the HOO group) and elevated by TDYA, a specific inhibitor for peroxisomal $\beta$-oxidation. CFB treatment caused diminished ketogenesis rate in HOO-fed rats, as shown in Fig. 4B. When octanoate was used as a substrate, there was no difference in the ketone body production (Fig. 4B). The results suggested that peroxisomal oxidation of erucic acid inhibited mitochondrial $\beta$-oxidation by blocking the entry of cytosolic long-chain fatty acids into mitochondria. To verify this, liver malonyl-CoA was measured, and HRO treatment remarkably increased liver malonyl-CoA content (by 162 and 119% versus the HOO and LRO groups, respectively), as further increased by the treatment of CFB and reduced by TDYA. Liver malonyl-CoA was also significant higher in the HOO+CFB group compared with the HOO group (by 127% versus the HOO group) (Fig. 4C). The results confirmed that the diminished mitochondrial FAO in livers of the rats fed HRO was due to elevated malonyl-CoA level, which specifically inhibited carnitine palmitoyltransferase-1a (CPT1a) and restricted the transport of long-chain fatty acid into mitochondria (18, 19). Liver malonyl-CoA decarboxylase (MCD) activity was not statistically significantly different among all the groups (Fig. 4D), suggesting the elevated malonyl-CoA formation as caused by erucic acid oxidation was due to increased generation of malonyl-CoA in liver. In the meantime, hepatic citrate content was not significantly altered by HRO feeding or CFB treatment, suggesting an extramitochondrial source of acetyl-CoA for malonyl-CoA synthesis (Fig. 4E). The results indicated that peroxisomal $\beta$-oxidation of erucic acid suppressed mitochondrial $\beta$-oxidation by stimulating malonyl-CoA formation, whereas increased oleic acid flux through peroxisomal $\beta$-oxidation in the HOO+CFB group exhibited similar effects as in the HRO group, which further suggested that stimulation of malonyl-CoA formation and suppression of mitochondrial fatty acid oxidation were a general phenomenon occurring when the fatty acid flux through peroxisomal $\beta$-oxidation was increased.

**Peroxisomal $\beta$-oxidation of erucic acid generated acetate as a precursor for cytosolic acetyl-CoA synthesis**

Because erucic acid is mainly oxidized in peroxisomes, the product of peroxisomal $\beta$-oxidation of erucic acid was analyzed. The addition of C22:1-CoA into liver homogenate led to dose-dependent generation of acetate, as completely blocked by pretreatment of TDYA-CoA, whereas the addition of C6-CoA, a
substrate for mitochondrial \( \beta \)-oxidation, contributed very little to acetate formation in liver homogenate (Fig. 5A). Incubation of purified peroxisomes with C22:1-CoA led to the release of acetate dose-dependently, as reduced by pretreatment of TDYA-CoA (Fig. 5B). The generation of acetate in peroxisomes was due to a specific acetyl-CoA hydrolase (ACOT12) in liver peroxisomes. Gene expression of ACOT12 was up-regulated in livers of the rats fed HRO and strongly induced by CFB treatment. D, peroxisomal ACOT12 activity increased in livers of the rats fed HRO and was further enhanced by CFB treatment, whereas peroxisomal CAT activity was very low in rat liver. E, peroxisomal acetyl-CoA content increased significantly in livers of the rats fed HRO and was further elevated after treatment of CFB and reduced by TDYA. F, liver acetate content increased significantly in the rats fed HRO, as further elevated by CFB, and was reduced by the treatment with TDYA. Results are mean ± S.E. (error bars); *, \( p < 0.05 \) by t test between paired conditions.

Peroxisomal oxidation of erucic acid enhanced acetyl-CoA synthetase (ACS) and acetyl-CoA carboxylase (ACC) activity by elevating cytosolic NADH/NAD\(^+\) ratio and inhibiting SIRT1 activity

The addition of C22:1-CoA into liver homogenates elevated the NADH/NAD\(^+\) ratio dose-dependently, as lowered by TDYA-CoA, whereas there was no alteration for C6-CoA (Fig. 6A). High NADH/NAD\(^+\) ratio suppressed the activity of SIRT1, a NAD\(^+\)-dependent deacetylase that plays a critical role in regulating fatty acid oxidation and synthesis, including the enzymes in cytosolic acetyl-CoA and malonyl-CoA generation. SIRT1 activity was then measured, and the addition of C22:1-CoA to rat liver homogenate significantly inhibited SIRT1 activity, as recovered by pretreatment of TDYA-CoA (Fig. 6B). Cytosolic NADH/NAD\(^+\) ratio was significantly higher in the livers of HRO feeding rats (by 135%, 87% versus normal and LRO-fed rats, respectively), as robustly elevated by CFB treatment (by 38% versus HRO control), and lowered by TDYA (Fig. 6C). SIRT1 activity was diminished in liver of the rats fed HRO (decreased by 27% versus LRO feeding) and further suppressed by the treatment of CFB (Fig. 6D). ACS expression was up-regulated in livers of the HRO-fed rats, as strongly induced by CFB treatment and lowered by TDYA (Fig. 6E). Liver ACS activity was significantly higher in rats fed HRO and further increased by CFB and decreased by TDYA (Fig. 6F). Cytosolic acetyl-CoA concentration was elevated by HRO feeding or CFB treatment as reduced by TDYA (Fig. 6G). Hepatic AMP-activated protein kinase (AMPK) activity was significantly reduced by HRO feeding or CFB treatment as elevated by TDYA (Fig. 6H). Liver ACC activity was significantly higher in HRO-feeding rats, as further increased by CFB and decreased by TDYA treatment (Fig. 6I). The results indicated that elevated cytosolic NADH/NAD\(^+\) ratio in HRO-fed rats suppressed the activity of SIRT1 and AMPK, which subsequently increased ACS and ACC activity, and stimulated

Figure 5. Peroxisomal oxidation of erucic acid generated appreciable acetate in liver. A, the addition of C22:1-CoA to liver homogenate led to dose-dependent generation of acetate, as inhibited by TDYA-CoA, and no significant generation of acetate with C6-CoA. B, incubation of isolated peroxisomes with C22:1-CoA or acetyl-CoA generated acetate dose-dependently, as blocked by pretreatment of TDYA-CoA. C, mRNA expression level of ACOT12 was up-regulated in livers of the rats fed HRO and strongly induced by CFB treatment. D, peroxisomal ACOT12 activity increased in livers of the rats fed HRO and was further enhanced by CFB treatment, whereas peroxisomal CAT activity was very low in rat liver. E, peroxisomal acetyl-CoA content increased significantly in livers of the rats fed HRO and was further elevated after treatment of CFB and reduced by TDYA. F, liver acetate content increased significantly in the rats fed HRO, as further elevated by CFB, and was reduced by the treatment with TDYA. Results are mean ± S.E. (error bars); *, \( p < 0.05 \) by t test between paired conditions.
hepatic malonyl-CoA formation from cytosolic acetate, thereby suppressing mitochondrial fatty acid oxidation.

**Discussion**

Chronic uptake of high-erucic-acid rapeseed oil causes hepatic steatosis in animals and possibly in humans (4, 5); however, for a long time, the mechanism linking erucic acid metabolism and lipid deposition has not been clear. The results in this study indicate that oxidation of erucic acid by peroxisomes increased malonyl-CoA generation in rat liver and suppressed mitochondrial fatty acid oxidation, and chronic intake of high-erucic-acid rapeseed oil led to diminished mitochondrial fatty acid oxidation and hepatic steatosis, which revealed a mechanism linking erucic acid and hepatic steatosis. The proposed mechanism is shown in Fig. 7. Excessive uptake of erucic acid in liver resulted in activation of PPARα, and the gene expressions involved in peroxisomal β-oxidation were extensively induced. Liver oxidation of erucic acid by peroxisomes led to increased generation of free acetate and elevated cytosolic acetyl-CoA. Peroxisomal oxidation also significantly elevated the redox state of cytosolic NADH/NAD⁺ and activated ACC via suppression of SIRT1, which stimulated the synthesis of malonyl-CoA and led to diminished mitochondrial fatty acid oxidation and hepatic steatosis.

This study established a cross-talk between peroxisomal and mitochondrial fatty acid oxidation in liver through the formation of malonyl-CoA, and peroxisomal oxidation of very long-chain fatty acid stimulated malonyl-CoA formation, thereby suppressing mitochondrial fatty acid oxidation. As the core molecule in fatty acid synthesis, malonyl-CoA was identified to be the molecule responsible for the inhibition of CPT1a, the critical enzyme in the transfer of long-chain fatty acids into mitochondria, thereby suppressing mitochondrial fatty acid oxidation and hepatic steatosis.

Although the peroxisomal β-oxidation system was discovered more than 40 years ago, the intrinsic relationship between the
Proposed mechanism by which peroxisomal oxidation of erucic acid suppressed mitochondrial fatty acid oxidation and led to hepatic steatosis.

Figure 7.

Peroxisomal and mitochondrial fatty acid oxidation systems are not fully established (20). It was proposed that this metabolism system was to handle excessive fatty acids that were left by mitochondrial fatty acid oxidation, which transferred the acetyl-CoA to mitochondria for final burning (21). However, there was evidence that peroxisomal β-oxidation did not contribute to significant ketone body formation. For example, the addition of palmitic acid to hepatocytes stimulated peroxisomal β-oxidation, whereas ketone bodies were not significantly increased (22). Our results suggested that peroxisomal β-oxidation of erucic acid suppressed mitochondrial fatty acid oxidation through increasing liver malonyl-CoA level, and specific inhibition of peroxisomal β-oxidation partly abolished the effects on malonyl-CoA generation and mitochondrial fatty acid oxidation as caused by erucic acid. We therefore proposed that one of the physiological functions of peroxisomal β-oxidation was to stimulate the formation of malonyl-CoA in liver through metabolism of endogenous substrates, thereby suppressing mitochondrial β-oxidation.

Very long-chain fatty acids have been well-accepted to be exclusively metabolized in peroxisomes (23), and our results confirmed that erucic acid was oxidized preferentially by the peroxisomal β-oxidation system. Mitochondria showed very low oxidative activity toward C22:1-CoA, which was in good agreement with previous reports (7, 8). Interestingly, the results indicated that the presence of erucic acid resulted in an adaptive elevation in peroxisomal β-oxidation capacity through activation of PPARα, which suggested that erucic acid might act as a potential ligand for PPARα because long-chain fatty acids were identified to be endogenous ligands for PPARα and triggered downstream transcription of the target genes, including the genes involved in peroxisomal β-oxidation (17, 24, 25), thereby accelerating peroxisomal turnover of erucic acid. It was reported that administration of PPARα activator stimulated hepatic fatty acid synthesis (26). Our results suggested that a regulatory mechanism existed for the control of mitochondrial fatty acid oxidation through peroxisomal metabolism of very long-chain fatty acids that was induced by PPARα.

Peroxisomes are not permeable to acetyl-CoA (27). It is well-accepted that there are two pathways for acetyl-group transfer from peroxisome to the cytosol. One way is to hydrolyze acetyl-CoA to acetate via peroxisomal acetyl-CoA thioesterase, and the other way is to transform the acetyl-CoA to acetyl-carnitine via CAT (28). We analyzed the final product of C22:1-CoA that was subjected to peroxisomal β-oxidation, and the results clearly indicated that peroxisomal oxidation of C22:1-CoA generated free acetic acid as the predominant product, as further confirmed in vivo, which was in good agreement with previous reports that peroxisomal fatty acid metabolism generated free acetic acid (29, 30). The formation of acetate from acetyl-CoA was attributed to high-level expression and activity of ACOT12 in rat liver. On the other hand, the activity of CAT was very low in rat liver, whereas its expression was high in heart and muscle (31). Cytosolic free acetate can barely be metabolized in liver because it can hardly be transformed to acetyl-CoA in mitochondria due to lack of a specific acetyl-CoA synthetase in liver mitochondria (32). The acetate that was released from peroxisomal β-oxidation of erucic acid was then used for the synthesis of cytosolic acetyl-CoA because a specific acetyl-CoA synthetase existed in the cytosol of rat liver (33, 34). The acetyl-CoA generated from peroxisome-released acetate would then be further transformed to malonyl-CoA by acetyl-CoA carboxylase (35). It is interesting to note that an acetyl-CoA synthetase was in the mitochondria of rat heart, suggesting that the acetate generated in heart may be used as an energy source (35). In this circumstance, we noted the fact that acetate generated from ethanol oxidation stimulated liver fatty acid synthesis and led to significant hepatic steatosis (36).

Unlike mitochondria, a respiration chain is absent in peroxisomes; however, it was reported that the redox state of pyridine nucleotide within the peroxisome and the cytosol are in equilibrium through lactate/pyruvate and glycerol phosphate shuttles (27, 37). Therefore, elevated erucic acid oxidation by peroxisomes would lead to increased NADH release from the peroxisomes to the cytosol and significantly increased cytosolic NADH/NAD⁺ ratio. High NADH/NAD⁺ ratio resulted in diminished activity of SIRT1, a NAD⁺-dependent deacetylase (38). The decreased SIRT1 activity may reduce the deacetylation of LKB1 and inhibit this kinase, which in turn inhibits AMPK (39, 40). SIRT1 and AMPK are known fuel-sensing molecules, and activation of the SIRT1/AMPK pathway plays a central role in regulating hepatic fatty acid metabolism (39). The diminished AMPK activity may further lead to activation of ACC, the key enzyme for malonyl-CoA synthesis. Inhibition of ACOX1, the rate-limiting enzyme in peroxisomal β-oxidation by a specific inhibitor TDYA significantly decreased the liver...
NADH/NAD\textsuperscript{+} ratio, which resulted in activation of AMPK and suppression of ACC via decreasing the deacetylation level to LKB1 and PGC-1\textalpha (12). It was reported that PPAR\alpha agonist clofibrate treatment significantly increased the NADH/NAD\textsuperscript{+} ratio in rats (41), indicating that peroxisomal \( \beta\)-oxidation was critical for the control of cytosolic NADH/NAD\textsuperscript{+} ratio. We also noted that ethanol oxidation in liver also caused a significant increase in the liver NADH/NAD\textsuperscript{+} redox state and suppressed mitochondrial \( \beta\)-oxidation and led to hepatic lipid deposition, as completely abolished by pyrazole, an inhibitor of alcohol dehydrogenase (36). Therefore, suppression of AMPK and activation of ACC by erucic acid were attributed at least in part to the elevation of the cytosolic NADH/NAD\textsuperscript{+} ratio and the diminished SIRT1 activity through peroxisomal oxidation of this very long-chain fatty acid.

Mitochondrial dysfunction has been well-accepted to play a critical role in the development of nonalcoholic fatty liver disease and related metabolic disorder (42). The results suggested that excessive oxidation of erucic acid in liver peroxisomes suppressed mitochondrial fatty acid oxidation, and specific inhibition of peroxisomal \( \beta\)-oxidation significantly attenuated erucic acid--induced depression in mitochondrial fatty acid oxidation and hepatic steatosis. Accumulation of hepatic lipid has been well-accepted to be a critical cause of insulin resistance (43). Therefore, the results in this research had clinical significance; excessive oxidation of erucic acid or endogenous very long-chain fatty acids by peroxisomes might turn out to be a pathological cause that leads to fatty liver and insulin resistance in our modern life. High-erucic-acid rapeseed oil as an edible oil is widely consumed in South China and India (44), and more than 150 million people in China suffer from fatty liver and diabetes (45). Although the clinical connection between high-erucic-acid rapeseed oil intake and metabolic diseases has not yet been established, we propose that chronic intake of high-erucic-acid rapeseed oil might lead to a high risk of liver steatosis and insulin resistance and strongly suggest low-erucic-acid rapeseed oil or olive oil as an edible oil instead of high-erucic-acid rapeseed oil in daily life (46–48). This might benefit the liver as well as the heart by reducing the harmful metabolites released from peroxisomal \( \beta\)-oxidation upon mitochondrial fatty acid oxidation. On the other hand, this mechanism is not confined to erucic acid, as long-chain fatty acids were ideal substrates for the peroxisomal \( \beta\)-oxidation system, and peroxisomal oxidation of long-chain fatty acids is significantly elevated under the conditions of obesity and diabetes (6). We therefore further propose that peroxisomal oxidation of exogenous or endogenous long-chain fatty acids might act as a common mechanism for fatty acid--induced hepatic steatosis and insulin resistance. Small molecules that specifically inhibit peroxisomal \( \beta\)-oxidation might be promising agents for treating fatty liver and related metabolic diseases as caused by excessive fatty acid metabolism through peroxisomal \( \beta\)-oxidation.

**Materials**

CoA sodium salt, malonyl-CoA, acetyl-CoA, C6-CoA, C16-CoA, Percoll, cytochrome c, 5,5-dithio-bis(2-nitrobenzoic acid) (DTNB) and defatted BSA were purchased from Sigma. CFB, octanoic acid, erucic acid, palmitic acid, and TDYA were from Tokyo Chemical Industry (Tokyo, Japan). The CoA thioesters of erucic acid (C22:1-CoA) and TDYA (TDYA-CoA) were prepared according to the method of Li et al. (49, 50). All other chemical reagents used were of analytical grade or better.

**Animal studies**

Male Sprague–Dawley rats were purchased from Slac Laboratory Animal Co. Ltd. (Changsha, China). Standard rodent diet (12% fat by calories), a high-rapeseed-oil diet containing 15% (w/w) rapeseed oil (55% fat by calories), and HOO containing 15% (w/w) olive oil (55% fat by calories) were supplied by Slac Laboratory Animal Co. Ltd. The rapeseed oil used in HRO contained 35% (w/w) erucic acid, whereas the rapeseed oil in LRO contained 2% (w/w) erucic acid. All animals were housed in single cage with free access to food and water under controlled temperature (22 °C) and light (12 h of light and 12 h of dark).

Male Sprague–Dawley rats at the age of 8 weeks (200–220 g) were exposed to standard, HOO, LRO, or HRO diet for 4 weeks. CFB (200 mg/kg) or TDYA (50 mg/kg) was administered to the indicated groups by gavage, once per day at 5 p.m. All rats were weighed every day at 5 p.m. An oral glucose tolerance test was performed on day 25. Glucose at 3 g/kg was introduced by gavage for each rat, with blood samples collected at the indicated time from tail vein, and blood glucose was determined by a glucometer (Lifescan, Johnson and Johnson). After the experiments, all of the rats were bled from the eyes and then sacrificed. Livers were removed quickly, weighed, and stored in liquid nitrogen immediately. The HOMA-IR index was calculated as described previously (51). All of the animal studies were approved by the Animal Care Committee of Hunan University of Science and Technology.

**Histological analysis**

Same parts of the right lobe were cut quickly from the livers of the killed rats and immediately fixed with 4% paraformaldehyde. Paraffin sections were prepared and cut into 5–7-μm-thick sections and stained by hematoxinlin-eosin. Hepatic steatosis was observed by an optical microscope, and four samples were used for each group to observe the lipid droplets in liver tissues.

**Fatty acid oxidation by isolated hepatocytes**

Hepatocytes of treated rats were prepared as described before (52). Cell viability was assessed by trypan blue exclusion and estimated to be more than 90%. Ketogenesis and CO\textsubscript{2} formation, as specific indicators for mitochondrial \( \beta\)-oxidation, were performed as described before with newly isolated intact hepatocytes after minor modification. Hepatocytes (1 × 10\textsuperscript{6}) were incubated under an atmosphere of O\textsubscript{2}/CO\textsubscript{2} (19:1) in Krebs–Henseleit bicarbonate medium, pH 7.4, containing 0.4 mM [1-\textsuperscript{14}C]palmitate or 0.8 mM [1-\textsuperscript{14}C]octanoate, 0.34 mM defatted BSA, and 1 mM carnitine. After incubation at 37 °C for 15 min, the reaction was terminated by the addition of ice-cold perchloric acid. After injection of hyamine hydroxide, the flask...
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was shaking for another 45 min to trap $^{14}$CO$_2$ for the radioactivity assay. The rate of mitochondrial fatty acid oxidation determined by the $^{14}$CO$_2$ formation was expressed as nmol of labeled carbon atoms/min/g of cell (wet weight). After neutralization and centrifugation, ketone bodies (sum of β-hydroxybutyrate and acetoacetate) in the supernatant were determined enzymatically (53).

**Isolation of liver subcellular fractions**

Mitochondria were isolated by differential centrifugation in 0.25 m sucrose as described previously (54), the mitochondrial pellet was washed three times and resuspended in the same medium at a concentration of ~40 mg/ml. The integrity of isolated mitochondria was examined by a commercial mitochondrial staining kit (Sigma). For the isolation of peroxisomes, the light mitochondrial (L) fraction after differential centrifugation was further isolated by a Percoll gradient as described before (55). 15 mg of L fraction sample was layered on 5 ml of 50% Percoll containing 250 mM sucrose, 2 mM Mops, 1 mM EGTA, and 0.1% (v/v) ethanol at pH 7.2. After centrifugation at 85,000 × g for 30 min on a Beckman Optima MAX-XP ultracentrifuge with an MLN80 rotor, the fractions were collected for the catalase activity assay. The pooled peak fractions were diluted with 250 mM sucrose and centrifuged at 35,000 × g for 15 min to recover sediment containing purified peroxisomes. For the preparation of cytosolic fraction, the post-light mitochondrial supernatant was first centrifuged at 27,000 × g for 15 min to pellet residual large organelles and finally centrifuged at 100,000 × g for 60 min to obtain a cytosolic fraction for the metabolite and enzyme assay.

The purity of isolated organelle was determined by marker enzyme activity assay: catalase for peroxisomes and cytochrome c oxidase for mitochondria (55). Purity of mitochondria and peroxisomes was more than 92%, whereas mitochondria or peroxisome contamination was less than 1% in purified peroxisome or mitochondria, facilitating reliable measurement of peroxisomal or mitochondrial FAO.

**Characteristics of the enzymes involved in liver β-oxidation**

Mitochondrial SCAD, MCAD, LCAD, and peroxisomal ACOX1 were expressed in Escherichia coli and purified by nickel metal affinity chromatography. Mitochondrial CPT1 was expressed in Saccharomyces cerevisiae and purified as described (56). Acyl-CoA dehydrogenases were measured using 2,6-dichloroindophenol as the final electron receptor (57). ACOX1 activity was assayed spectrophotometrically by directly measuring H$_2$O$_2$ formation (12). CPT1 activity was assayed by measuring the formation of free CoA by a DTNB method (56).

**Measurement of peroxisomal and mitochondrial β-oxidation**

Mitochondrial β-oxidation was determined spectrophotometrically by the method of Osmundsen and Bremer (58), with 100 μM palmitoyl-CoA (C16-CoA) or erucyl-CoA (C22:1-CoA) as a substrate. Peroxisomal β-oxidation was assayed by acyl-CoA-dependent NAD$^+$ reduction in the presence of KCN as reported by Lazarow (59), with 100 μM C22:1-CoA as a substrate.

**Studies of erucic acid metabolism in liver homogenate**

The reaction mixture contained 50 mM Hepes, pH 7.4, 1 mM NAD$^+$, 5 mM pyruvate, 2 mM CoA, 5 mM ATP, 0.5 mg/ml defatted BSA, 10 mM MgCl$_2$, 50 μM FAD, 20 mg of liver homogenate, and 0.5 mM C$_6$-CoA or C22:1-CoA at a concentration of 0.025, 0.05, 0.1, and 0.2 mM, respectively. In some cases, just 5 min before C22:1-CoA treatment, 100 μM TDYA-CoA was added to inhibit peroxisomal oxidation of erucic acid. After reaction at 37°C for 30 min, NADH/NAD$^+$ redox state, acetate content, hydrogen peroxide generation, and SIRT1 activity were analyzed.

**Studies of erucic acid metabolism in peroxisome**

The reaction mixture contained 130 mM KCl, 20 mM Hepes (pH 7.2), 0.1 mM EGTA, 0.5 mM NAD$^+$, 0.1 mM NADP$^+$, 1 mM CoA, 0.1 mM DTT, 5 mM MgCl$_2$, 5 mM ATP, 1 unit of lactate dehydrogenase (Sigma), 5 mM pyruvate, 1 unit of catalase (Sigma), 1 mg/ml defatted BSA, 0.2 mM acetyl-CoA or C22:1-CoA at a concentration of 0.025, 0.05, 0.1, and 0.2 mM, respectively, and 2 mg of isolated peroxisomes in a total volume of 1 ml (60). 100 μM TDYA-CoA was added for the inhibition of peroxisomal β-oxidation as indicated. After incubation at 37°C for 30 min, the reaction was terminated by the addition of ice-cold perchloric acid (70%, w/v). Acetate contained in neutralized supernatant was then measured.

**Quantitative real-time PCR**

Total RNA was extracted from liver tissues with TRIzol reagent (Life Technologies, Inc.). RNA was reverse-transcribed with standard reagents (High Capacity Reverse Transcription Kits, Applied Biosystems) using random primers. Complementary DNA was amplified in a 7500 Fast Real-time PCR System using 2× SYBR Green Supermix (Applied Biosystems). The primers used were as follows: ACOX1, 5'-TGGAGAGGCTCCTAGC-TATGG-3' (forward) and 5'-GGTTTACCAGCCTGTAAG-3' (reverse); Acyl-CoA synthetase, 5'-GGTCTTAGGATAA-AGGCTGACGT-3' (forward) and 5'-TATGG-3' (reverse); ABCD1, 5'-ATCG-3' (forward) and 5'-GACTTC-3' (reverse); L-BP, 5'-AGGCTCTGCTCAGTGCTCGTCCGTG-3' (reverse); thiolase, 5'-AGGCTCTGCTCAGTGCTCGTCCGTG-3' (reverse); ABCD1, 5'-ATCG-3' (forward) and 5'-GACTTC-3' (reverse); L-BP, 5'-AGGCTCTGCTCAGTGCTCGTCCGTG-3' (reverse); 18S rRNA, 5'-CGTCTGCTCAGTGCTCGTCCGTG-3' (reverse) and 5'-CGTCTGCTCAGTGCTCGTCCGTG-3' (reverse). mRNAs expression levels normalized to 18S rRNA were expressed using the comparative ΔCT method.

**Biochemical analysis**

Plasma TAG was determined by a commercial kit according to the manufacturer’s instructions (Wako, Osaka, Japan). Liver long-chain acyl-CoAs were extracted and determined by the method of Tubbs and Garland (61). Liver TAG were extracted
by the method of Bligh and Dyer (62) and determined with a commercial kit (Wako, Osaka, Japan). Liver hydrogen peroxide, NADH/NAD⁺ ratio, citrate, and acetate were determined by commercial kits (Sigma). Peroxisomal and cytosolic acetyl-CoA content was measured by a fluorometric assay kit from Sigma, and cytosolic concentration of acetyl-CoA was calculated according to the water space of the cytosolic fraction (63). The cytosolic NADH/NAD⁺ ratio was determined by an indirect method by analyzing the concentration of lactate and pyruvate, which are in equilibrium with free NADH and NAD⁺ in a lactate dehydrogenase system (64). Liver malonyl-CoA was analyzed by HPLC as described previously (65). Plasma insulin was measured by a rat/mouse insulin ELISA kit from Merck Millipore (Billerica, MA, USA). Protein concentration was measured by a Bio-Rad DC protein assay kit (Hercules, CA, USA).

Peroxisomal acetyl-CoA thioesterase (ACOT12) was measured by a DTNB assay using isolated peroxisomes with 100 µM acetyl-CoA (66). Peroxisomal CAT activity was assayed as described before using 100 µM acetyl-carnitine (67). Activity of malonyl-CoA decarboxylase was measured enzymatically by a coupled reaction with malate dehydrogenase and citrate synthase (68). The reaction solution contained 0.1 M Tris-HCl (pH 8.0), 0.5 mM DTT, 0.6 mM NAD⁺, 1 mM malate, malate dehydrogenase (74 units), 300 µM malonyl-CoA, citrate synthase (1.7 units), and 0.3 µg of liver homogenate in a total volume of 1 ml. SIRT1 deacetylase activity of rat liver was evaluated by the Cyclex SIRT1/Sir2 deacetylase fluorometric assay kit (CyclLex, Nagano, Japan) according to the manufacturer’s guidance. SIRT1 activity was expressed as relative fluorescence/mg of protein (arbitrary units). ACS activity was determined spectrophotometrically as described previously (32). Liver AMPK activity was measured spectrophotometrically by a commercial kit (Genmed Scientific Inc., Arlington, MA, USA). Liver ACC activity was measured by a commercial kit (Solarbio, Beijing, China).

Statistical analysis

Data are presented as mean ± S.E. n = 6–8 for all of the groups. The significance of differences was evaluated using Student’s t test by SPSS 18.0. p < 0.05 was considered statistically significant.

Data availability

All data are contained within the article.

Author contributions—X. C. and P. L. data curation; X. C., L. S., P. L., K. C., T. G., Z. C., and J. Z. formal analysis; X. C., L. S., S. D., P. L., K. C., T. G., Z. C., and J. Z. investigation; X. C., L. S., S. D., Z. C., and J. Z. methodology; L. S. validation; S. D. and J. Z. supervision; J. Z. conceptualization; J. Z. funding acquisition; J. Z. writing–original draft; J. Z. project administration.

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Abbreviations—The abbreviations used are: FAO, fatty acid oxidation; PPARα, peroxisome proliferator activator receptor α isoform; CFB, clofibrate; TDYA, 10,12-tricosadiynoic acid; CPT1, carnitine palmitoyltransferase-I; SCAD, short-chain acyl-CoA dehydrogenase; MCAD, medium-chain acyl-CoA dehydrogenase; ACOX1, acyl-CoA oxidase-1; LCAD, long-chain alcohol dehydrogenase; C6, hexanoyl; TAG, triacylglyceride; ACOT12, acetyl-CoA hydrolase; CAT, carnitine acetyltransferase; L-BP, L-bifunctional protein; thiolase, peroxisomal 3-oxoacyl-CoA thiolase; ABCD1, peroxisomal ATP-binding cassette transporter D; ACS, acetyl-CoA synthetase; ACC, acetyl-CoA carboxylase; SIRT1, silent information regulator 1; AMPK, AMP-activated protein kinase; HOMA-IR, homeostasis model assessment of insulin resistance; MCD, malonyl-CoA decarboxylase; HRO, high-erucic-acid rapeseed oil diet; LRO, low-erucic-acid rapeseed oil diet; HOO, high-olive-oil diet; DTNB, 5,5-dithio-bis(2-nitrobenzoic acid).

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