Peroxisome-generated succinate induces lipid accumulation and oxidative stress in the kidneys of diabetic mice

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Diabetes normally causes lipid accumulation and oxidative stress in the kidneys, which plays a critical role in the onset of diabetic nephropathy; however, the mechanism by which dysregulated fatty acid metabolism increases lipid and reactive oxygen species (ROS) formation in the diabetic kidney is not clear. As succinate is remarkably increased in the diabetic kidney, and accumulation of succinate suppresses mitochondrial fatty acid oxidation and increases ROS formation, we hypothesized that succinate might play a role in inducing lipid and ROS accumulation in the diabetic kidney. Here we demonstrate a novel mechanism by which diabetes induces lipid and ROS accumulation in the kidney of diabetic animals. We show that enhanced oxidation of dicarboxylic acids by peroxisomes leads to lipid and ROS accumulation in the kidney of diabetic mice *via* the metabolite succinate. Furthermore, specific suppression of peroxisomal β-oxidation improved diabetes-induced nephropathy by reducing succinate generation and attenuating lipid and ROS accumulation in the kidneys of the diabetic mice. We suggest that peroxisome-generated succinate acts as a pathological molecule inducing lipid and ROS accumulation in kidney, and that specifically targeting peroxisomal β-oxidation might be an effective strategy in treating diabetic nephropathy and related metabolic disorders.

Diabetes has been well known to induce lipid accumulation and oxidative stress in the kidney, which plays a critical role in the development of diabetic nephropathy (1–4). However, the mechanism by which dysregulated glucose and fatty acid oxidation (FAO) in diabetes causes ectopic lipid deposition and excessive formation of reactive oxygen species (ROS) in kidney is not fully demonstrated. Although malonyl-CoA plays a critical role in controlling mitochondrial FAO, we noted the fact that hepatic and kidney malonyl-CoA level is significantly reduced under the condition of diabetes or fasting (5, 6), indicating that malonyl-CoA may not play a role in inducing lipid accumulation in the diabetic kidney. Therefore, identification of the pivotal molecule that might suppress mitochondrial fatty acid oxidation in the kidney of diabetic animals will be critical.

To explore such a molecule, we focused on succinate, a unique molecule that exhibited multiple physiological functions (7–9). The cross talk between succinate oxidation and lipid accumulation in kidney is not established so far; however, we noted the well-known concept that excessive succinate oxidation causes robust reduction of mitochondrial NAD^+ by blocking the electron flow from the NADH to the cytochromes (10–12), which causes accumulation of the intermediates in fatty acid oxidation and feedback suppression of mitochondrial FAO (13–15). Besides, excessive oxidation of succinate in respiration chain also leads to considerable ROS formation within mitochondria (16, 17). As succinate content increased remarkably in the kidney of the diabetic animals (18), we therefore hypothesized that succinate might play a role in inducing lipid and ROS accumulation in the diabetic kidney and causing related nephropathy in diabetes.

This study investigated the role of succinate in regulating mitochondrial FAO and ROS homeostasis in the kidney of streptozotocin (STZ)-induced diabetic mice and explored the potential mechanism by which diabetes stimulated succinate generation and induced lipid and ROS accumulation in kidney.

Results

Accumulation of succinate caused suppression of mitochondrial FAO and increased ROS formation

As expected, the content of succinate increased significantly in the kidney of STZ-induced diabetic mice, as shown in Figure 1A, which was in agreement with previous report (18). To investigate whether succinate might affect mitochondrial fatty acid oxidation in the kidney, β-oxidation was assayed in the isolated mitochondria from mouse kidney cortex in the presence of succinate. Hexanoic acid (C6), a specific substrate for mitochondrial FAO, was used as a surrogate for mitochondrial β-oxidation and at a concentration of 2 mM in the incubation medium. The results indicated that addition of 2 mM succinate to incubation medium suppressed mitochondrial oxidation of C6, as indicated by accumulation of C6-CoA, which was abolished by pretreatment of malonate, an inhibitor for succinate dehydrogenase (SDH) (Fig. 1B). β-hydroxybutyrate (βOHB)/acetoacetate (AcAc) ratio as a measure of intramitochondrial $NADH/NAD^+$ ratio was determined, and the results suggested that addition of succinate remarkably elevated βOHB/AcAc ratio in mitochondria (Fig. 1C). Elevation in mitochondria $NADH/NAD^+$ ratio caused accumulation of 3-hydroxyacyl-CoA (3-OH-CoA) and 2-enoyl-CoA in the

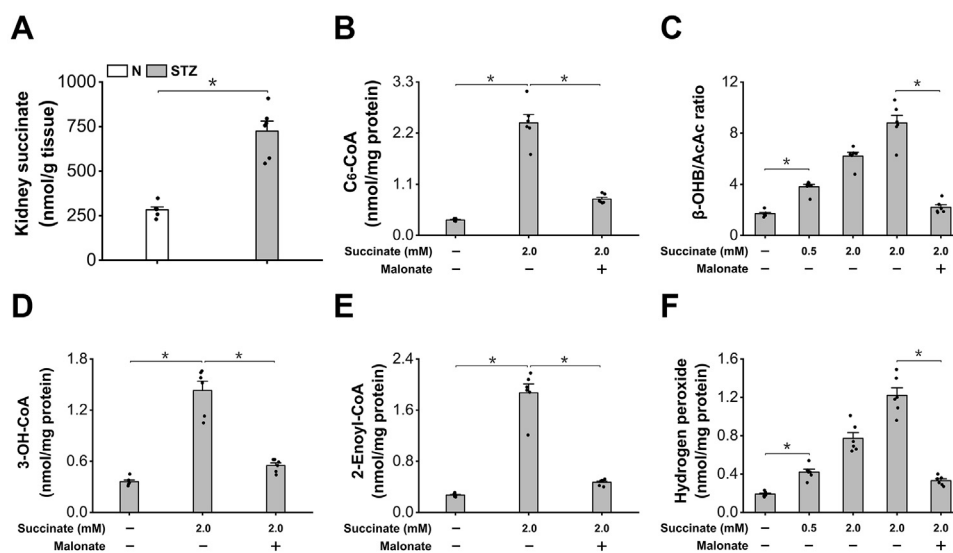* For correspondence: Jia Zeng, zengj@hnu.edu.cn.

Figure 1. Succinate acts as a suppressor for mitochondrial β-oxidation. *A*, the content of succinate increased significantly in the kidney of STZ-induced diabetic mice. *B*, in the presence of 2 mM hexanoic acid (C6), addition of 2 mM succinate to the isolated mitochondria of kidney cortex caused accumulation of C6-CoA, as reduced by pretreatment of malonate. *C*, addition of 2 mM succinate to the isolated mitochondria remarkably elevated βOHB/AcAc ratio. *D* and *E*, in the presence of C6, addition of 2 mM succinate caused accumulation of (*D*) 3-hydroxyacyl-CoA (3-OH-CoA) and (*E*) 2-enoyl-CoA intermediates in the isolated mitochondria, as abolished by pre-treatment of malonate. *F*, addition of succinate significantly increased hydrogen peroxide formation in the isolated mitochondria, as reduced by pretreatment of malonate. Mean ± SEM, n = 6, *$p < 0.05$ by *t* test between paired conditions. STZ, streptozotocin.

presence of C6 substrate (Fig. 1, *D* and *E*), which are strong feedback inhibitors for 2-enoyl-CoA hydratase and acyl-CoA dehydrogenase, respectively (19–21). Pretreatment of malonates completely abolished the effects of succinate on the NADH redox state and accumulation of FAO intermediates (Fig. 1, *C–E*), Therefore, the diminished mitochondrial β-oxidation as caused by succinate was attributed to the elevation in mitochondrial $NADH/NAD^+$ redox state and accumulation of the reaction intermediates. Addition of succinate to the isolated mitochondria also significantly stimulated hydrogen peroxide formation, as abolished by pretreatment of malonate (Fig. 1*F*). The results indicated that accumulation of succinate caused suppression mitochondrial β-oxidation and increased ROS generation, which might play a role in diabetes-induced lipid and ROS accumulation in kidney.

Fatty acid ω-oxidation and peroxisomal β-oxidation were induced in the kidney of diabetic mice

As succinate is not cell-membrane-permeable (22), the increased succinate in the diabetic kidney should be generated within kidney cortex. It should be noted that the formation of succinate in the tricarboxylic acid (TCA) cycle is strictly regulated at the α-ketoglutarate step (23), therefore, the elevated succinate was not likely due to enhancement of TCA cycle. SDH activity was also measured, the results indicated that diabetes did not cause significant decrease in SDH activity (Fig. 2*A*). Therefore, the increased succinate in the kidney of the diabetic mice was not attributed to decreased succinate turnover in the TCA cycle. To identify the extramitochondrial source of succinate, we proposed that endogenous dicarboxylic acids (DCAs), the product of fatty acids subjected to ω-oxidation (24, 25), might be a potential source for succinate

because succinate is the ultimate product of DCAs subjected to β-oxidation (26–28). It is generally accepted that DCAs are oxidized exclusively by peroxisomal β-oxidation system, and the kidney is the primary site for metabolism of DCAs (28–30). Our results confirmed that DC-CoAs were exclusively metabolized in peroxisomes from mouse kidney cortex, as reflected by the specific activity of mitochondrial long-chain acyl-CoA dehydrogenase (LCAD) and peroxisomal ACOX-1 toward DC_{12}-CoA, mitochondrial LCAD showed no catalytic activity to DC_{12}-CoA, while high activity for DC-CoA was observed in ACOX-1, pretreatment of 100 μM 10,12-tricosadiynoyl-CoA (TDYA-CoA) (31), a specific inhibitor for peroxisomal β-oxidation strongly suppressed peroxisomal β-oxidation of DC_{12}-CoA, as shown in Figure 2*B*. We further analyzed peroxisomal succinyl-CoA level, and the results indicated that peroxisomal succinyl-CoA increased remarkably in the kidney cortex of STZ-induced diabetic mice (Fig. 2*C*), which well supported that the increased generation of succinate in the kidney of diabetic mice was of peroxisomal origin. Diabetes resulted in elevation in plasma free fatty acids and increased uptake of fatty acids by the kidney, as shown in Figure 2, *D* and *E*, which plays a critical role in inducing fatty acid ω-oxidation and peroxisomal β-oxidation. The mRNA expressions of both fatty acid ω-oxidation and peroxisomal β-oxidation were extensively induced in the kidney cortex of the STZ-induced diabetic mice (Fig. 2*F*). Dicarboxylyl-CoA (DC-CoA) synthetase activity and peroxisomal β-oxidation were enhanced significantly in the kidney of STZ-induced diabetic mice, which led to accelerated turnover of endogenous DCAs (Fig. 2, *G* and *H*). Therefore, induction of peroxisomal DCA oxidation might lead to increased succinate generation in the diabetic kidney. We therefore proposed that peroxisomal β-oxidation might play a role in inducing lipid and ROS
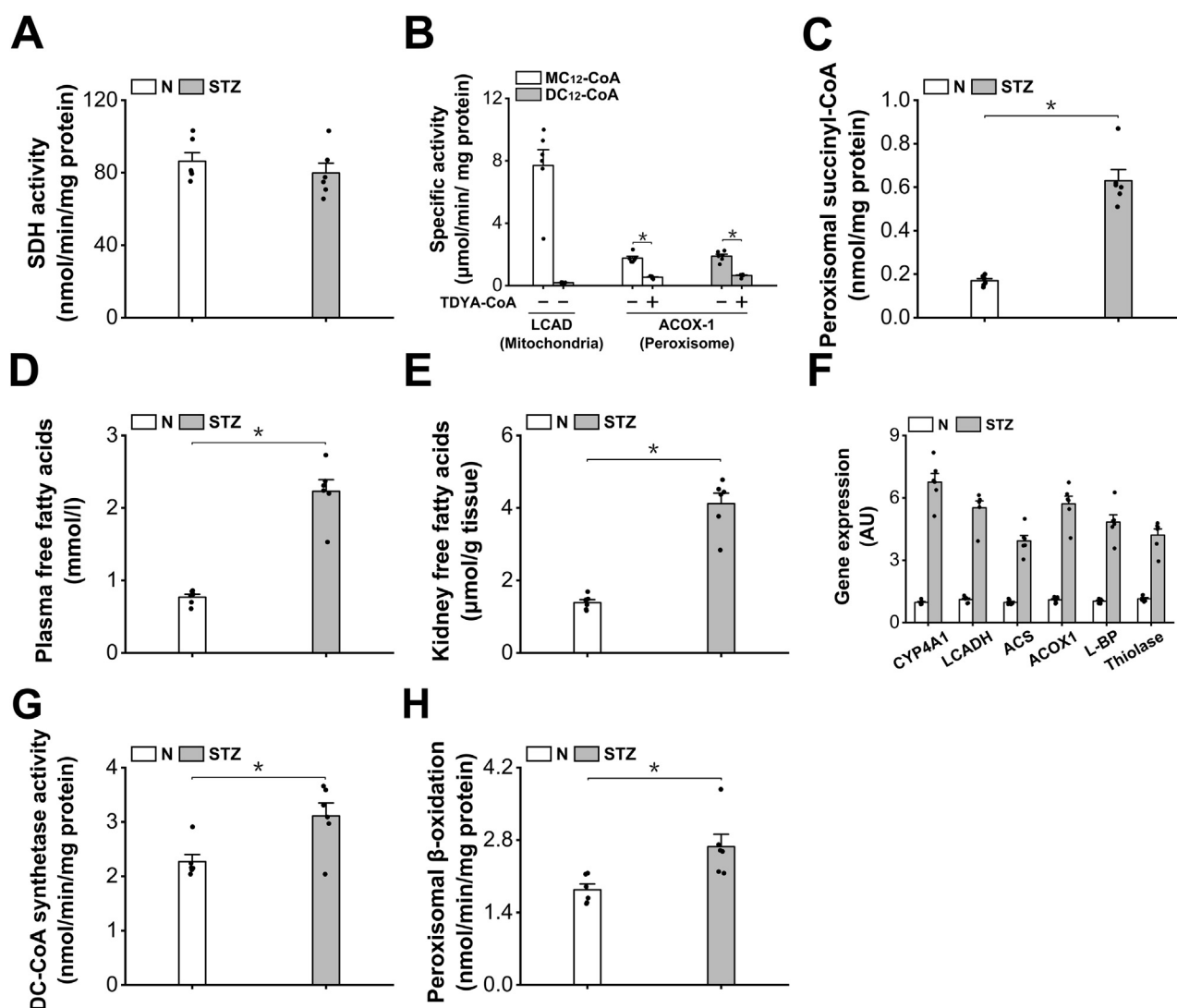
Figure 2. Peroxisomal β-oxidation of dicarboxylic acids was upregulated in the kidney cortex of STZ-induced diabetic mice. *A*, succinate dehydrogenase (SDH) activity was not altered in the kidney of STZ-induced diabetic mice. *B*, oxidation of MC_{12}-CoA (CoA thioester of dodecanoic acid) and DC_{12}-CoA (mono-CoA thioester of dodecanedioic acid) by mitochondrial LCAD and peroxisomal ACOX-1, pretreatment of 100 μM TDYA-CoA suppressed peroxisomal β-oxidation. *C*, peroxisomal succinyl-CoA increased significantly in the kidney cortex of STZ-induced diabetic mice. *D*, plasma free fatty acids were elevated in the diabetic mice. *E*, kidney free fatty acids increased significantly in the STZ-induced diabetic mice. *F*, gene expressions of enzymes in fatty acid ω-oxidation and peroxisomal β-oxidation were up-regulated in the kidney cortex of STZ-induced diabetic mice. *G*, the activity of DC-CoA synthetase increased significantly in the kidney cortex of STZ-induced diabetic mice. *H*, peroxisomal β-oxidation was enhanced in the kidney cortex of STZ-induced diabetic mice. Mean ± SEM, n = 6, *$p < 0.05$ by *t* test between paired conditions. STZ, streptozotocin; TDYA-CoA, 10,12-tricosadiynoyl-CoA.

accumulation in the diabetic kidney through the metabolite succinate.

Peroxisomal β-oxidation of DCAs generated succinate

We used isolated peroxisomes from mouse kidney cortex to determine whether peroxisomal β-oxidation of long-chain DCAs generated succinate. The results indicated that addition of DC_{12}-CoA (mono-CoA thioester of dodecanedioic acid) to peroxisomes led to dose-dependent generation of succinyl-CoA and succinate, as completely blocked by pretreatment of TDYA-CoA, a specific inhibitor for peroxisomal β-oxidation (Fig. 3, *A* and *B*). Peroxisomal generation of succinyl-CoA was attributed to the very much low activity of peroxisomal carnitine octanoyltransferase (COT) toward DC-CoAs compared

with monocarboxylyl-CoA (MC-CoAs) (Fig. 3*C*), which led to accumulation of succinyl-CoA after β-oxidation of DC_{12}-CoA. A specific succinyl-CoA thioesterase (ACOT4) was present in mouse kidney peroxisomes with a K_M of 13 μM (32), the mRNA expression and activity of ACOT4 in the kidney cortex of STZ-induced diabetic mice were upregulated significantly compared with the normal control (Fig. 3, *D* and *E*), which greatly accelerated succinate formation from succinyl-CoA in the kidney cortex of diabetic animals.

DCAs suppressed mitochondrial FAO and increased ROS generation through the metabolite succinate

To determine whether peroxisomal oxidation of DCAs might cause suppression of mitochondrial fatty acid oxidation

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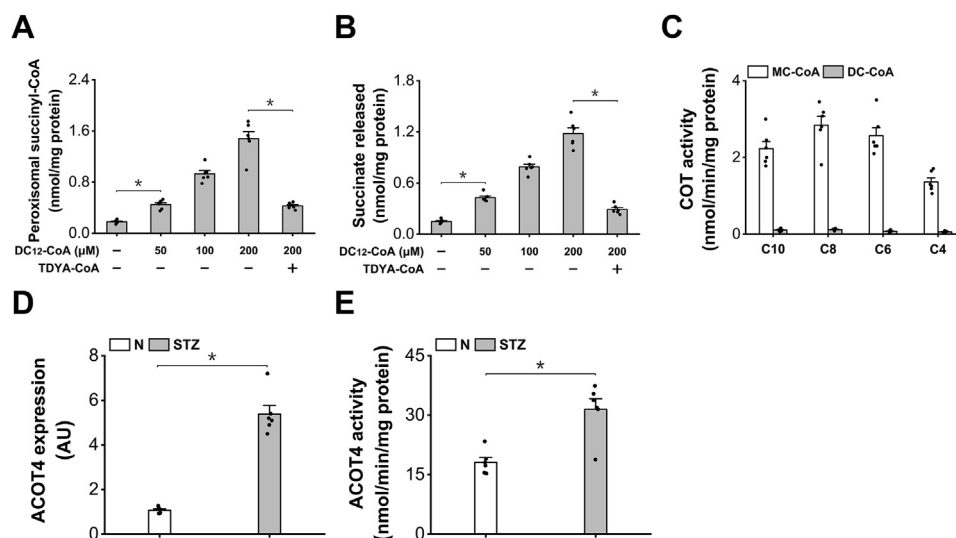*J. Biol. Chem.* (2022) 298(3) 101660 **3**

Figure 3. Peroxisomal oxidation of dicarboxylic acids generated succinate. *A* and *B*, addition of DC$_{12}$-CoA to the isolated peroxisome led to dose-dependent generation of (*A*) succinyl-CoA and (*B*) succinate, as reduced by the treatment with TDYA-CoA. *C*, the activity of peroxisomal carnitine octanoyltransferase (COT) in the kidney cortex toward DC-CoA and MC-CoA. *D*, mRNA expression of succinyl-CoA thioesterase (ACOT4) was upregulated in the kidney cortex of STZ-induced diabetic mice compared to the normal group. *E*, the activity of ACOT4 increased significantly in the kidney cortex of diabetic mice. Mean ± SEM, n = 6, *$p < 0.05$ by *t* test between paired conditions. TDYA-CoA, 10,12-tricosadiynoyl-CoA.

and increase ROS formation in kidney, we used dodecanedioic acid (DCA$_{12}$), a long-chain DCA to specifically increase peroxisomal β-oxidation of DCA, and 10,12-tricosadiynoic acid (TDYA), a specific inhibitor for peroxisomal β-oxidation to inhibit peroxisomal β-oxidation. Addition of DCA$_{12}$ into the homogenate of mouse kidney cortex led to dose-dependent formation of succinate (Fig. 4*A*), as reduced by pretreatment of TDYA. The presence of DCA$_{12}$ remarkably elevated βOHB/AcAc ratio (Fig. 4*B*), which caused diminished mitochondrial β-oxidation of C6 and accumulation of C6-CoA, as abolished by the treatment with malonate or TDYA (Fig. 4*C*). DCA$_{12}$ treatment also significantly stimulated hydrogen peroxide formation in the kidney homogenate and reduced by the treatment of malonate or TDYA (Fig. 4*D*). The results indicated that long-chain DCA suppressed mitochondrial fatty acid oxidation and increased ROS generation through the metabolite succinate.

Peroxisome-generated succinate induced lipid and ROS accumulation in the diabetic kidney

To investigate the role of peroxisome-generated succinate in kidney lipid and ROS homeostasis of the diabetic animals, TDYA, a specific inhibitor for ACOX-1, was administered to suppress peroxisomal β-oxidation of DCAs. Peroxisomal β-oxidation in the kidney cortex increased after feeding with DCA$_{12}$ and suppressed after treatment with TDYA (Fig. 5*A*). DCA$_{12}$ treatment significantly increased peroxisomal succinyl-CoA and succinate content in the kidney of STZ-induced diabetic mice, as reduced by the treatment of TDYA (Fig. 5, *B* and *C*).

βOHB/AcAc ratio was significantly higher in the kidney cortex of the diabetic mice and lowered by the treatment of TDYA, DCA$_{12}$ treatment led to further increase in βOHB/AcAc ratio in the kidney cortex of the diabetic mice, while

MCA$_{12}$ treatment caused no significant alteration in βOHB/AcAc ratio (Fig. 6*A*). DCA$_{12}$ treatment caused accumulation of 3-OH-CoA and 2-enoyl-CoA intermediates in the kidney cortex of STZ-induced diabetic mice, which significantly increased long-chain acyl-CoA (LC-CoA) level in the kidney cortex, while no significant changes after treatment with
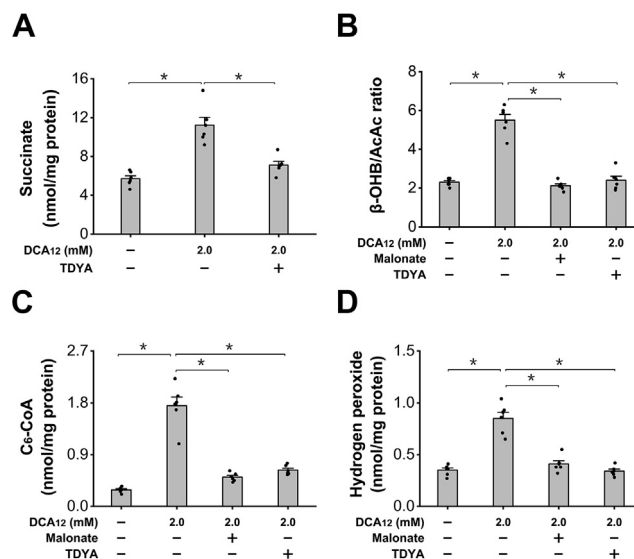

Figure 4. DCA$_{12}$ treatment suppressed mitochondrial β-oxidation and increased ROS formation *via* the metabolite succinate. *A*, addition of DCA$_{12}$ into the kidney cortex homogenate led to dose-dependent formation of succinate, as reduced by pretreatment of TDYA. *B*, DCA$_{12}$ treatment significantly elevated βOHB/AcAc ratio, as lowered by pretreatment of malonate or TDYA. *C*, addition of DCA$_{12}$ caused accumulation of C6-CoA in the homogenate of kidney cortex, as reduced by the treatment with malonate or TDYA. *D*, DCA$_{12}$ treatment significantly increased hydrogen peroxide formation in the kidney cortex homogenate, as reduced by the treatment of malonate or TDYA. Mean ± SEM, n = 6, *$p < 0.05$ by *t* test between paired conditions. DCA$_{12}$, dodecanedioic acid; TDYA, 10,12-tricosadiynoyl.
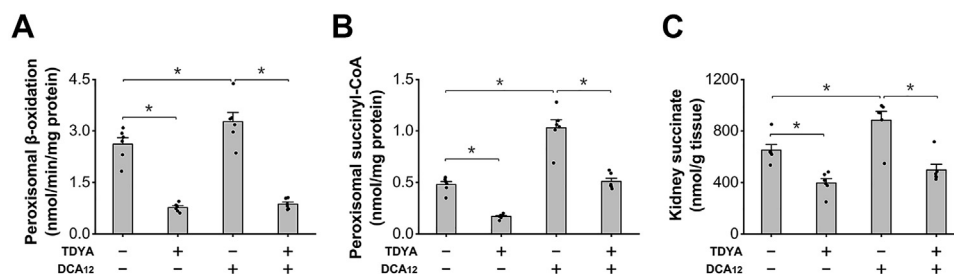
Figure 5. Excessive β-oxidation of DCAs stimulated succinate generation in STZ-induced diabetic mice. *A*, DCA_{12} treatment significantly enhanced peroxisomal β-oxidation in the kidney cortex of STZ-induced diabetic mice, as suppressed after treatment with TDYA. *B*, DCA_{12} treatment significantly increased peroxisomal generation of succinyl-CoA in the kidney cortex of diabetic mice, as reduced by the treatment of TDYA. *C*, DCA_{12} treatment increased succinate content in the kidney of STZ-induced diabetic mice, as reduced by the treatment of TDYA. Mean ± SEM, n = 6, *$p < 0.05$ by *t* test between paired conditions. DCA, dicarboxylic acid; TDYA, 10,12-tricosadiynoyl.

MCA_{12} (Fig. 6, *B–D*). Administration of TDYA significantly reduced the content of 3-OH-CoA and 2-enoyl-CoA intermediates and decreased LC-CoA content in the kidney cortex of the diabetic mice. TAG level in kidney cortex was significantly higher in the diabetic mice and reduced by the treatment of TDYA, while DCA_{12} feeding remarkably

increased TAG content in the kidney of the diabetic mice, and no alteration after treatment with MCA_{12} (Fig. 6*E*). TDYA treatment also lowered kidney ratio of the diabetic mice, and DCA_{12} treatment caused a further increase in kidney ratio (Fig. 6*F*). Kidney malonyl-CoA was significantly lower in the STZ-induced diabetic mice compared with the normal group,
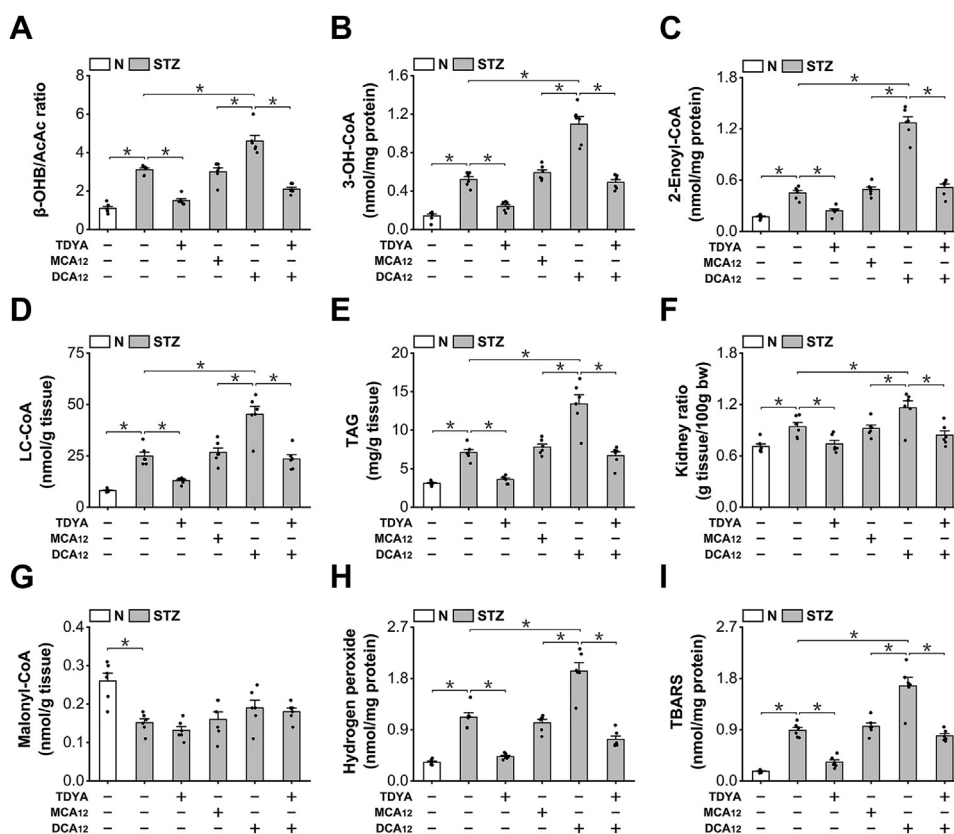

Figure 6. Peroxisome-generated succinate through oxidation of DCAs induced lipid and ROS accumulation in STZ-induced diabetic mice. *A*, βOHB/AcAc ratio was significantly higher in the kidney cortex of the diabetic mice and lowered by the treatment of TDYA, while DCA_{12} treatment led to further increase in βOHB/AcAc ratio in the kidney of STZ-induced diabetic mice. *B* and *C*, DCA_{12} treatment caused further accumulation of (*B*) 3-OH-CoA and (*C*) 2-enoyl-CoA intermediates in the kidney cortex of the diabetic mice, as reduced after treatment with TDYA. *D*, DCA_{12} treatment caused further accumulation of long-chain acyl-CoA (LC-CoA) in the kidney cortex of the diabetic mice, as reduced by the treatment of TDYA. *E*, TAG level was significantly higher in the kidney cortex of STZ-induced diabetic mice, DCA_{12} treatment caused further increase in TAG content in the diabetic kidney, as reduced by the treatment of TDYA. *F*, DCA_{12} treatment caused a further increase in the kidney ratio of STZ-induced diabetic mice, which was lowered by TDYA. *G*, kidney malonyl-CoA was significantly lower in the diabetic mice compared with the normal group, while no significant alterations after treatment with TDYA or DCA_{12}. *H*, DCA_{12} feeding remarkably increased hydrogen peroxide formation in the kidney of STZ-induced diabetic mice, as reduced by the treatment of TDYA. *I*, TBARS increased significantly in the kidney of the diabetic mice, as further increased by the treatment of DCA_{12} and reduced after treatment with TDYA. Mean ± SEM, n = 6, *$p < 0.05$ by *t* test between paired conditions. DCA, dicarboxylic acid; TBARS, thiobarbituric-acid-reactive substances; TDYA, 10,12-tricosadiynoyl.

while no significant alterations after treatment with TDYA, DCA_{12} or MCA_{12} (Fig. 6G). Hydrogen peroxide increased significantly in the kidney cortex of the diabetic mice compared with the normal mice, as reduced by TDYA, DCA_{12} treatment caused a further increase in hydrogen peroxide generation in the diabetic kidney and no changes after treatment with MCA_{12} (Fig. 6H). Thiobarbituric-acid-reactive substances (TBARS) as a marker for oxidative stress increased significantly in the kidney cortex of STZ-induced diabetic mice compared with the normal group, as further increased by the treatment of DCA_{12} and reduced after treatment with TDYA (Fig. 6I). The results provided evidence that peroxisome-generated succinate through oxidation of DCAs caused accumulation of lipid and ROS in the kidney cortex of STZ-induced diabetic mice.

Peroxisome-generated succinate plays a role in the development of diabetic nephropathy

Previous reports indicated that accumulation of lipids induced upregulation of expression of the growth factors such as TGF-β and VEGF (33–35), which have been shown to play an important role in mediating glomerulosclerosis and proteinuria and led to the development of diabetic nephropathy (33–36). Accumulation of ROS will also result in oxidative stress and induce expressions of the inflammatory cytokines such as tumor necrosis factor-α (TNFα) and interleukin-6 (IL-6) in the diabetic kidney (37–39). As peroxisome-generated succinate caused lipid and ROS accumulation in the diabetic kidney, we proposed that this molecule should play a role in the development and onset of diabetic nephropathy. The results indicated that DCA_{12} treatment significantly increased mRNA expression level of TGF-β and VEGF in the kidney of the diabetic mice, as decreased by the treatment with TDYA

(Fig. 7, A and B). The mRNA expressions of TNF-α and IL-6 in the kidney cortex increased significantly in the diabetic mice compared with the normal mice, as was further elevated by the treatment of DCA_{12} and decreased by TDYA (Fig. 7, C and D). Urine albumin-to-creatinine (UAC) ratio as a marker for early diabetic nephropathy was significantly higher in the diabetic mice compared with normal group, as further elevated after treatment with DCA_{12} and lowered significantly by TDYA (Fig. 7E). The results suggested that peroxisome-generated succinate induced lipid and ROS accumulation and played a role in the onset of diabetic nephropathy, while specific inhibition of peroxisomal β-oxidation reduced renal succinate generation and improved diabetes-induced nephropathy.

Discussion

This research demonstrated a novel mechanism by which diabetes induced lipid and ROS accumulation in kidney, the proposed mechanism was shown in Figure 8. Elevation in plasma FFA in diabetes results in increased uptake of fatty acids in the kidney, and fatty acid ω-oxidation and peroxisomal β-oxidation are induced in the kidney cortex of diabetic mice. Accelerated peroxisomal β-oxidation of DCAs stimulates succinate formation and remarkably increases mitochondrial $NADH/NAD^+$ ratio, which suppresses mitochondrial β-oxidation and generated considerable hydrogen peroxide, and leads to lipid and ROS accumulation in kidney, and ultimately led to the development and onset of diabetic nephropathy. Specific inhibition of fatty acid ω-oxidation or peroxisomal β-oxidation reduced succinate generation and improved lipid and ROS homeostasis in the diabetic kidney.

Succinate has been well known to exhibit multiple physiological functions, including metabolism, signal transduction, ROS generation, and tumorigenesis (7–9). However, whether
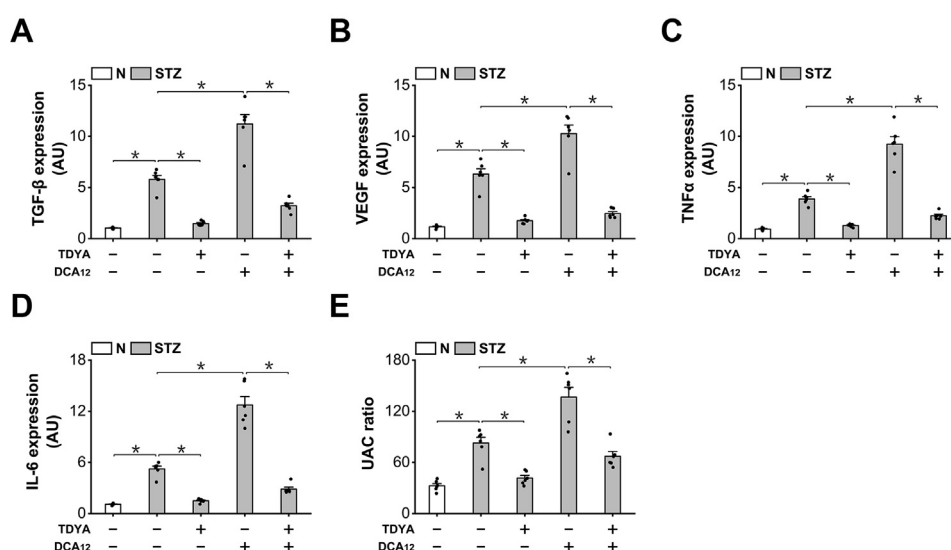

Figure 7. Excessive generation of succinate by peroxisomes induced expressions of growth factors and inflammation cytokines in the kidney of STZ-induced diabetic mice. *A* and *B*, mRNA expressions of (*A*) TFG-β and (*B*) VEGF increased significantly in the kidney of STZ-induced diabetic mice treated with DCA_{12}, as reduced by the treatment of TDYA. *C* and *D*, mRNA expressions of (*C*)TNFα and (*D*) IL-6 increased significantly in the kidney of STZ-induced diabetic mice treated with DCA_{12}, as reduced by the treatment of TDYA. *E*, urinary albumin creatinine (UAC) ratio was elevated significantly in STZ-induced diabetic mice treated with DCA_{12}, which was lowered by TDYA. Mean ± SEM, n = 6, *$p < 0.05$ by *t* test between paired conditions. DCA_{12}, dodecanedioic acid; STZ, streptozotocin; TDYA, 10,12-tricosadiynoyl.

Diabetes

↓

Kidney FFA ↑

↓ ω-oxidation ↑

Kidney DCA ↑

↓ Peroxisomal β-oxidation ↑ ⊣ **TDYA**

Kidney succinate ↑

Mitochondrial NADH/ NAD+ ↑

⊣

Mitochondrial β-oxidation

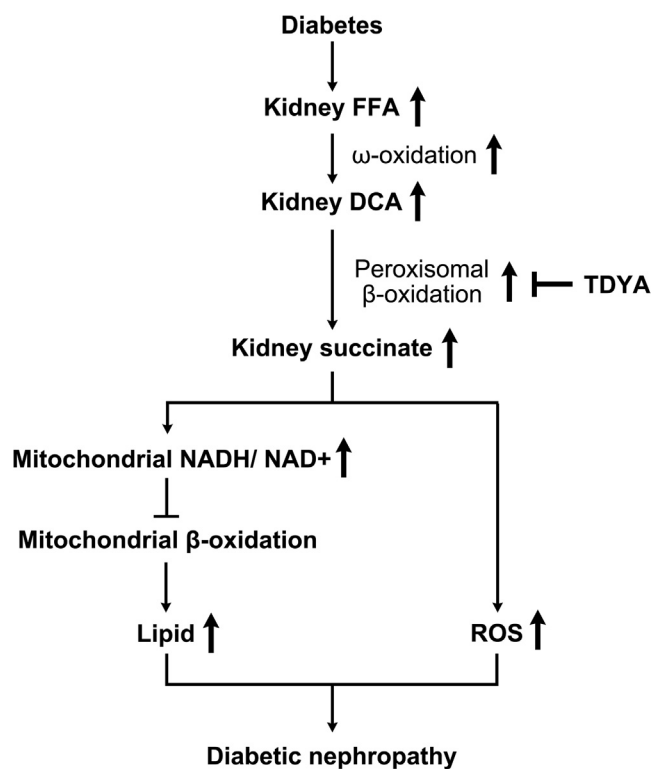**Lipid** ↑   **ROS** ↑

Diabetic nephropathy

Figure 8. Proposed mechanism by which diabetes induced lipid and ROS accumulation in the kidney of diabetic animals.

this molecule might have a regulatory role in fatty acid oxidation is not fully demonstrated, especially in the kidney when tissue level of succinate is high. As mitochondrial β-oxidation is under the control of the redox state of NAD+ (40), elevation in mitochondrial NADH/NAD+ ratio leads to suppression of mitochondrial fatty acid oxidation (14–16). Excessive succinate oxidation causes robust reduction of mitochondrial NAD^+ by blocking the electron flow from the NADH to the cytochromes as extensively studied by Chance and Krebs (10–12), which caused accumulation of 3-OH-CoA/ 2-enoyl-CoA intermediates and suppression of mitochondrial fatty acid oxidation in the liver (15, 30). In this study, succinate acted as a suppressor for mitochondrial β-oxidations and accumulation of succinate led to lipid accumulation in the kidney of diabetic animals, which well confirmed the mechanism for succinate control of mitochondrial β-oxidation in the kidney.

It is well accepted that excessive generation of ROS causes oxidative stress, which plays an important role in the development of diabetic nephropathy (16, 17). It was reported that the respiration chain was the major site for production of ROS and might lead to oxidative stress (17); however, the role of succinate in stimulating ROS generation and inducing oxidative stress in the kidney is never illustrated. Accumulation of succinate has been well known to generate considerable hydrogen peroxide and superoxide radicals in mitochondria (16, 17) and might lead to oxidative stress and relative tissue injury. As succinate increased remarkably in the kidney of the diabetic animals, this molecule might play a critical role in inducing oxidative stress and related tissue injury. In this

study, we observed that the increased formation of succinate in the diabetic kidney was attributed at least in part to peroxisomal oxidation of long-chain DCAs, reducing the supply of DCAs or suppressing peroxisomal β-oxidation significantly decreased succinate generation and reduced ROS formation in the kidney of diabetic animals.

Fatty acid ω-oxidation and DCAs were discovered in the kidney and urine of animals and humans in the 1930s (24). As fatty acid ω-oxidation is induced and considerable DCAs were observed in the kidney of diabetic animals (Figs. 2*F* and 5*A*), endogenous DCAs might play a physiological role under certain conditions such as diabetes. Although endogenous DCA has been discovered for a long time, the physiological functions of these kinds of fatty acid are not clear. It is proposed that the generation of DCAs may facilitate the oxidation of fatty acids (25). DCAs are also considered to be gluconeogenic precursors through generation of succinate in animals (41, 42). It was reported that administration of long-chain DCAs to alloxan diabetic rats rapidly and robustly decreased plasma ketone body in diabetic animals (41), indicating that endogenous DCAs might play a role in regulating mitochondrial β-oxidation. Our previous report confirmed that endogenous DCAs negatively regulated mitochondrial FAO and led to hepatic lipid accumulation through elevation in mitochondrial NADH/NAD+ redox state in the liver of the fasting rats (30). This study demonstrated a potential physiological function of endogenously generated DCAs in the kidney and suggested that a role of fatty acid ω-oxidation was to provide substrates for metabolism in peroxisomes for the purpose of generating succinate, which is increased significantly in the kidney and urine of the diabetic animals. Therefore, increases in DCA generation will negatively regulate mitochondrial fatty acid oxidation by elevating mitochondrial NADH/NAD+ ratio and increase ROS formation in the kidney of the diabetic animals through the metabolite succinate.

Peroxisomal β-oxidation system in mammalians was discovered in the 1970s (43); however, the physiological roles of this fatty acid oxidation system in lipid and ROS homeostasis in animals are not fully demonstrated. It was proposed that this metabolism system was to handle excessive fatty acids that left by mitochondrial fatty acid oxidation, which transferred the acetyl-CoA to mitochondria for final burning (44). On the other hand, the acetyl-CoA that generated in peroxisomal FAO might be used for biosynthesis of fatty acids or cholesterol (45, 46). Recently, it was reported that peroxisomal β-oxidation plays a role in inducing hepatic steatosis and oxidative stress in high-fat diet fed rats by stimulating formation of malonyl-CoA and increasing hydrogen peroxide formation (31, 47). Induction of peroxisomal β-oxidation also induced lipid accumulation in the liver of the fasting animals by stimulating DCA oxidation (30). It was reported that the acetyl-CoA derived from hepatic peroxisomal β-oxidation inhibits autophagy and promoted hepatic steatosis through activation of mTORC1 (48). Most of the studies on peroxisomal β-oxidation are concentrated in the liver; however, as the enzymes in peroxisomal FAO are also highly expressed in the kidney cortex and induced under the condition of diabetes,

this metabolism system might play a role in regulating lipid and ROS metabolism in kidney. This research demonstrated a cross talk between peroxisomal β-oxidation and lipid accumulation in the kidney of diabetic animals that peroxisomal oxidation of endogenous DCAs suppressed mitochondrial β-oxidation and caused lipid and ROS accumulation through the metabolite succinate, which serves as a novel mechanism for diabetes-induced renal dysfunction and nephropathy. As we know, this is the first report demonstrating a potential pathogenic role of peroxisomal β-oxidation in the kidney.

Both lipids and ROS are important factors causing renal dysfunction and related nephropathy (1–4). Accumulation of ROS and increased oxidative stress induce expressions of a series of inflammatory cytokines such as TNFα and interleukin-6 (IL-6) in the diabetic kidney, which plays a critical role in the initiation and development of diabetic nephropathy. In the meantime, it is also reported that excessive lipids deposition upregulated the expression of the growth factors such as TGF-β, PAL-1, and VEGF, which have been shown to play an important role in mediating glomerulosclerosis and proteinuria (37–39). This study demonstrated a novel pathogenic mechanism by which diabetes induces glomerulosclerosis and proteinuria as caused by lipid and ROS in the kidney, which suggests that succinate serves as a pathogenic molecule inducing lipid and ROS accumulation in the kidney and might play a critical role in onset of diabetic nephropathy. As fatty acid w-oxidation and peroxisomal β-oxidation are induced in the kidney under the condition of diabetes, accelerated DCA turnover will result in excessive succinate formation in the kidney of the diabetic animals. Therefore, the FFA-DCA-succinate axis might serve as a mechanism for diabetes-induced kidney dysfunction through inducing upregulation of the growth factors such as TGF-β, PAL-1, and VEGF and inflammatory cytokines. The results also suggested the pathogenic nature of endogenous dicarboxylic acids as accumulated in the kidney of the diabetic animals and humans, which was used as substrates for generation of succinate by peroxisomal β-oxidation. We suggest that urine or kidney level of DCAs and succinate serve as potential hallmarks of diabetes-induced metabolic disorder for clinical diagnosis.

A succinate receptor GPR91 is highly expressed in the kidney, and succinate has been shown to stimulate renin release from the kidney *via* GPR91 signal pathway and causes activation of Renin-Angiotensin System (RAS) (18, 49). It is proposed that RAS activation in diabetes mellitus is a core abnormality that leads to many complications of the disease, including proteinuria, and renal tissue injury and hypertension (49). Therefore, we suggest that excessive generation of succinate by peroxisomal oxidation of DCAs might also lead to activation of the RAS and cause chronic kidney diseases and hypertension. It will be of interest to investigate whether peroxisome-generated succinate plays a role in diabetes-induced alteration in renin secretion and hypertension.

This mechanism was verified by specific inhibition peroxisomal β-oxidation of DCAs (Figs. 5–7). Administration of a specific inhibitor for peroxisomal β-oxidation to the diabetic mice significantly enhances mitochondrial fatty acid oxidation and reduced lipid and ROS level in the kidney of diabetic mice. Clinical data indicate that short-chain dicarboxylic acids and succinate excretion in urine increase remarkably in the diabetic individuals (50, 51), short-chain DCAs are the products of endogenous long-chain DCAs subjected to β-oxidation in peroxisomes and should be mainly originated from the kidney of the diabetic patients; therefore, clinical evidence indicated that peroxisomal β-oxidation of endogenous DCAs should be markedly induced in the kidney of diabetic individuals. It is suggested that small molecules that specifically target fatty acid ω-oxidation or peroxisomal β-oxidation (*e.g.*, TDYA) might be promising agents in treating diabetes-induced nephropathy by reducing peroxisomal generation of succinate, which significantly decreases lipid level and reduces ROS formation in the diabetic kidney.

Experimental procedures

Materials

Acyl-CoAs (C4:0, C6:0, C8:0, C10:0, C12:0), coenzyme A sodium salt, malonyl-CoA, succinyl-CoA, succinate, malonate, Percoll, streptozotocin (STZ), 5,5-dithio-bis(2-nitrobenzoic acid) (DTNB), PEG 1500, and defatted bovine serum albumin (BSA) were purchased from Sigma. 10,12-tricosadiynoic acid (TDYA) and dodecanedioic acid (DCA$_{12}$) were from Tokyo Chemical Industry. The mono-CoA thioesters of dodecanedioic acid (DC$_{12}$-CoA), sebacic acid (DC$_{10}$-CoA), suberic acid (DC$_8$-CoA), and adipic acid (DC$_6$-CoA) were enzymatically prepared by a microsomal acyl-CoA synthetase and purified by high-performance liquid chromatography as previously described (30). All other chemical reagents used were of analytical grade or better.

Animal studies

Male C57BL/6 mice at the age of 8 weeks were obtained from Slac Laboratory Animal Co. Ltd. All the mice were housed in single cage with free access to food and water under controlled temperature (22 °C) and light. Diabetic mellitus was induced by administration of streptozotocin (70 mg/kg, i.p.) to the mice. Two weeks after the injection, the blood glucose level was determined by tail vein bleeding using a glucometer, and the mice with overt hyperglycemia (plasma glucose>400 mg/dl) were used for the experiments. All the diabetic mice were fed standard rodent diet (12% calories from fat) throughout the experiment. For the study of DCA on fatty acid and ROS metabolism in the kidney, DCA$_{12}$ (5%, w/w) was mixed in the diet and fed to the diabetic mice for 4 weeks, and diet mixed with dodecanoic acid (MCA$_{12}$) (5%, w/w) was used as a control. For TDYA intervention, TDYA was administered to indicated groups at a dose of 100 mg/kg by gavage twice daily for 4 weeks. Normal group was fed standard rodent diet throughout the experiment. After the experiments, all the mice were bled from eyes and then sacrificed, kidney samples were removed quickly and stored in liquid nitrogen immediately. All the animal studies were approved by the Animal Care Committee of Hunan University of Science and Technology.

Isolation of subcellular fractions

Tissue homogenates were prepared according the method of Antonenkov (52), and the kidney cortex was homogenized at 2 °C in the isolation medium containing 0.25 M sucrose, 10 mM MOPS, pH 7.4, 1 mM EDTA, 0.1% (vol/vol) ethanol, and 0.1 mM PMSF.

Mitochondria from mouse kidney cortex were isolated by differential centrifugation in 0.25 M sucrose performed as described previously (53), the mitochondrial pellet was washed three times in 0.25 M sucrose at 2 °C and finally suspended in the same medium at a concentration of ∼30 mg/ml for subsequent analysis. For the isolation of peroxisomes, light mitochondrial fraction (L) after differential centrifugation was further isolated by a Percoll gradient according the method of Neat and Antonenkov (52, 54). Twelve milligrams of L fraction sample was layered on 5 ml of a 50% (v/v) solution of Percoll containing 250 mM sucrose, 12% (wt/vol) PEG 1500, 2 mM Mops, 1 mM EGTA, and 0.1% (v/v) ethanol at pH 7.2. After centrifugation at 85,000g for 30 min on a Beckman Optima MAX-XP ultracentrifuge with an MLN80 rotor, the fractions were collected for catalase activity assay. The pooled peak fractions were diluted with 250 mM sucrose and centrifuged at 35,000g for 15 min to recover sediment containing purified peroxisomes.

The quality of the isolated mitochondrial and peroxisomal preparations was determined by measuring marker enzyme activities in the purified organelles according to the method as described previously (54), as shown in Table S1.

Studies of succinate or DCA$_{12}$ on mitochondrial β-oxidation

The reaction mixture contained 20 mM Hepes, pH 7.4, 110 mM KCl, 2 mM MgCl$_2$, 2 mM potassium phosphate, 2 mM ATP, 1.5 mg/ml defatted BSA, 50 μM FAD, 10 mg mitochondrial proteins or tissue homogenate, and 2 mM succinate or DCA$_{12}$. In some cases, just 5 min before succinate treatment, addition of 2 mM malonate to the reaction mixture completely inhibits succinate dehydrogenase activity, or 200 μM TDYA inhibits peroxisomal β-oxidation. After reaction at 37 °C for 20 min, mitochondrial β-oxidation, βOHB/AcAc ratio, and the reaction intermediates were analyzed.

Generation of succinyl-CoA and succinate from DC-CoAs

DC$_{12}$-CoA was used as substrate for generation of succinyl-CoA and succinate by the isolated peroxisomes from mouse kidney cortex. The reaction mixture contained 130 mM KCl, 20 mM Hepes (pH 7.2), 0.025% (v/v) Triton X-100, 0.1 mM EGTA, 0.5 mM NAD$^+$, 1 mM CoA, 0.1 mM dithiothreitol, 5 mM MgCl$_2$, 1 U lactate dehydrogenase (sigma), 5 mM pyruvate, 1 U catalase (sigma), 10 μg/ml antimycin, 1 mg/ml of defatted BSA, DC$_{12}$-CoA at a concentration of 50, 100, and 200 μM respectively, and 2 mg of isolated peroxisomes in a total volume of 1 ml. Occasionally, 100 μM TDYA-CoA was added to the reaction mixture for the inhibition of peroxisomal β-oxidation. After reaction for 30 min at 37 °C, the reaction was stopped by addition of ice-cold perchloric acid (70%, w/v),

succinyl-CoA and succinate contained in the neutralized supernatant were then measured.

Measurement of 3-OH-CoA and 2-enoyl-CoA intermediates of β-oxidation

3-OH-CoA and 2-enoyl-CoA in the isolated mitochondria or tissue samples were assayed by the coupled reaction of malic dehydrogenase and glutamic-oxaloacetic transaminase as described previously (55).

Quantitative real-time PCR

Total RNA was extracted from kidney cortex with TRIzol reagent (Life Technologies Corporation. RNA was reverse-transcribed with standard reagents using random primers. Complementary DNA was amplified in a 7500 Fast Real-time PCR System using 2×SYBR Green Supermix (Applied Biosystems). The following primers were used: CYP4A1, 5′- CCCGACACAGCCACTCATTC -3′ (F) and 5′- CCTT CAGCTCATTCATGGCAACT -3′ (R); ACOX-1,5′- TGGAGA GCCCTCAGCTATGG -3′ (F) and 5′- CGTTTCACC GCCTCGTAAG -3′ (R); LCADH, 5′- GGCTGGTTAAGTGA TCTCGTGAT -3′ (F) and 5′- TCTCCACCAAAAAGAGGC TAATG -3′(R); ACS, 5′- GGCTCTAGGAGTAAAGGCTGA CGT -3′ (F) and 5′- TCCTTTCGTTCTAGCTAGCTCCGT -3′(R); L-BP, 5′- AAATACAGAGATACCAGAAGCCG -3′ (F) and 5′- AAGAATCCCCAGTGTGACTTC -3′(R); Thiolase, 5′- CCTGACATCATGGGCATCG-3′ (F) and 5′- AGTCAG CCCTGCTTTCTGCA -3′(R); ACOT4, 5′- ATGCTTCGACA TCCAAAGGT -3′ (F) and 5′- GGAAGCCATGATCAGACAG AC-3′(R); TGF-β, 5′- CGCCATCTATGAGAAAACCAA -3′ (F) and 5′- AAGGTAACGCCAGGAATTGTT-3′ (R); VEGF, 5′- CAAACCTCACCAAAGCCAGC -3′ (F) and 5′- CACAG TGAACGCTCCAGGAT -3′(R); TNF-α, 5′- ATGGCC TCCCTCTCATCAGT -3′ (F) and 5′- GCAGCCTTGTCCC TTGAAGA -3′(R); IL-6, 5′- TCCTCTGGTCTTCTGGAGT ACC -3′ (F) and 5′- TGGTCCTTAGCCACTCCTTCTG -3′(R). 18S rRNA, 5′-GTTATGGTCTTTGGTCGC-3′ (F) and 5′-CGTCTGCCCTATCAACTTTC-3′ (R). mRNA expression levels normalized to 18S rRNA were expressed using the comparative delta CT method.

Biochemical analysis

Plasma FFA and TAG were determined by commercial kits (Wako). Total lipid from kidney cortex was extracted by the method of Bligh and Dyer (56), and triglycerides were determined by a commercial kit (Wako). Tissue β-hydroxybutyrate (βOHB) and acetoacetate (AcAc) were determined enzymatically according to the method as described previously (57). Kidney malonyl-CoA was analyzed by HPLC as described previously (58). Kidney hydrogen peroxide and thiobarbituric acid reactive substances (TBARS) were measured by assay kits from Sigma. Urine albumin and creatinine were measured with a mouse Albuwell enzyme-linked immunosorbent assay (ELISA) kit and a Creatinine Companion kit (Exocell, Inc.), results were expressed as urine albumin creatinine (UAC) ratio (μg/mg). Protein concentration was measured by Bio-Rad DC

protein assay kit (Hercules). Succinate dehydrogenase (SDH) activity was assayed according to the method of Ackrell (59). Succinate was assayed by succinate dehydrogenase method as described previously (60). Peroxisomal succinyl-CoA was assayed based on the reaction of DTNB with CoA liberated from succinyl-CoA in the presence of succinyl-CoA synthetase and monitored the absorbance at 412 nm (61). The standard assay system contained 10 mM $MgCl_2$, 2 mM GDP, 0.05 mM DTNB, 5 mM potassium phosphate, and 100 mM Hepes, pH 7.2. After preincubation for 5 min, the reaction was started by 1 U of succinyl-CoA synthetase. Dicarboxylyl-CoA (DC-CoA) synthetase activity was measured according to the method of Vamecq (62), with 1 mM DCA_{12} as substrate. Mitochondrial LCAD activity was determined spectrophotometrically by the method of Furuta (63), In total, 100 μM MC_{12}-CoA was used as substrate. Peroxisomal ACOX-1 activity was assayed spectrophotometrically by the method as described previously (31), with 100 μM MC_{12}-CoA or DC_{12}-CoA as substrate. Peroxisomal β-oxidation was assayed by acyl-CoA dependent NAD^+ reduction in the presence of KCN as developed by Lazarow PB (64), with DC_{12}-CoA as substrate. Peroxisomal succinyl-CoA thioesterase (ACOT4) was measured spectrophotometrically by a DTNB assay with isolated peroxisomes (65). Carnitine octanoyltransferase (COT) was assayed spectrophotometrically by following the release of CoA from mono-carboxyyl-CoAs and DC-CoAs at different chain length as described previously (66), 2 mM carnitine was used in the assays.

Statistic

Data are presented as mean ± SEM. The significance of the differences in mean values was evaluated using Student's t test. $p < 0.05$ was considered statistically significant.

Data availability

All data are available in the manuscript.

Conflict of interest—The authors declare that there is no conflict of interest with the content of this article.

Abbreviations—The abbreviations used are: βOHB, β-hydroxybutyrate; 3-OH-CoA, 3-hydroxyacyl-CoA; AcAc, acetoacetate; BSA, bovine serum albumin; COT, carnitine octanoyltransferase; DC-CoA, Dicarboxylyl-CoA; DCA, dicarboxylic acid; DCA12, dodecanedioic acid; DTNB, 5,5-dithio-bis(2-nitrobenzoic acid); ELISA, enzyme-linked immunosorbent assay; FAO, fatty acid oxidation; IL-6, interleukin-6; LC-CoA, long-chain acyl-CoA; LCAD, long-chain acyl-CoA dehydrogenase; MC-CoA, mono-carboxylyl-CoA; ROS, reactive oxygen species; SDH, succinate dehydrogenase; STZ, streptozotocin; TBARS, thiobarbituric-acid-reactive substances; TCA, tricarboxylic acid; TDYA, 10,12-tricosadiynoic acid; TNFα, tumor necrosis factor-α; UAC, urine albumin-to-creatinine.

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