Deleterious action of FA metabolites on ATP synthesis: possible link between lipotoxicity, mitochondrial dysfunction, and insulin resistance

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INSULIN RESISTANCE IS A CHARACTERISTIC feature of both type 2 diabetes mellitus and obesity (13). The etiology of insulin resistance is multifactorial and involves both genetic and environmental factors (4, 13, 14). Obesity and physical inactivity are among the principal environmental risk factors associated with insulin resistance. Both obese and lean insulin-resistant individuals manifest multiple disturbances in free fatty acid (FFA) metabolism, including increased day-long plasma FFA concentrations, an elevated basal rate of lipolysis, and impaired suppression of plasma FFA concentration by insulin (18, 19, 31, 40). A large body of evidence suggests that elevated plasma FFA levels play a pivotal role in the pathogenesis of insulin resistance. The plasma FFA concentration strongly correlates with whole body insulin sensitivity, measured with the insulin clamp technique (18, 19), and experimental elevation of plasma FFA concentration in insulin-sensitive subjects causes a dose-dependent decrease in whole body insulin sensitivity, associated with impaired insulin signaling in skeletal muscle (6). Although a causal role for dysregulated FFA metabolism in the development of insulin resistance is well recognized and has been referred to as “lipotoxicity” (5), the intracellular mechanisms by which elevated plasma FFA levels cause insulin resistance remain to be elucidated. Although increased skeletal muscle triglyceride content correlates closely with insulin resistance (26, 35, 36), triglycerides themselves are inert and do not cause insulin resistance. However, triglycerides are in a state of constant turnover, leading to the production of several lipid metabolites, including fatty acyl (FA)-coenzyme A (CoA), and ceramide, which through activation of serine kinases interfere with the insulin-signaling pathway and lead to a state of insulin resistance (5, 6, 17, 22, 33, 37, 45, 50).

The mechanism(s) responsible for the increase in intracellular lipid metabolites remain unclear. Insulin-resistant individuals have a reduced rate of fat oxidation compared with insulin-sensitive individuals (23), and decreased mitochondrial fat oxidative capacity could lead to an increase in intracellular fat content. Lowell and Shulman (29) suggested that a mitochondrial defect in insulin-resistant individuals could lead to an increase in intramyocellular fat content and cause insulin resistance. In support of this hypothesis, Morino et al. (33) and Petersen et al. (38) demonstrated that insulin-resistant offspring of two diabetic parents had a 35% reduction in muscle ATP synthesis rate, measured with 31P magnetic resonance spectroscopy. Petersen et al. (39) also demonstrated that the insulin resistance of aging was accompanied by an increase in intramuscular fat content, which was associated with an ~40% reduction in mitochondrial oxidative phosphorylation activity. These studies provide evidence for an association between increased intramuscular lipid content, mitochondrial dysfunction, and insulin resistance. However, it remains to be established what is the cause, and what is the effect, and which lipid metabolites are responsible for the insulin resistance. Elevation of the plasma FFA concentration in young healthy individuals produces a skeletal muscle mitochondrial defect that is similar to that observed in insulin-resistant individuals (9). Furthermore, in a previous study (42), we demonstrated that, in lean insulin-sensitive individuals, a physiological elevation in plasma...
FFA concentration increased intramyocellular FA-CoA levels and downregulated the expression of multiple genes encoding mitochondrial proteins, including those involved in oxidative phosphorylation. Increased levels of FFAs and their metabolites, e.g., FA-CoAs, have previously been reported to exert inhibitory actions on various mitochondrial functions (7, 8, 20, 27, 28, 46, 48, 49). Therefore, we postulate that a rise in intramyocellular FFA metabolite concentration, e.g., FA-CoA, represents a potential mechanism responsible for the acquired defect in mitochondrial function. In the present study, we investigated the effect of a physiological increase in fatty acid metabolites (palmitoyl carnitine, palmitoyl-CoA, and oleoyl-CoA) on mitochondrial ATP synthesis rate in mitochondria isolated from human and mouse skeletal muscle.

**METHODS**

**Chemicals**

Unless stated otherwise, all chemicals used in this study were obtained from Sigma.

**Animal Studies**

**Animals.** All mice used in this study were from the C57B6/J background and housed in the vivarium of the Audie L. Murphy Veterans Affairs Hospital. Mice were between 6 and 7 mo of age. Animals were anesthetized and killed by cervical dislocation, and mitochondria immediately were isolated as described below. All procedures were approved by the subcommittee for animal studies at the Audie L. Murphy Veterans Affairs Hospital.

**Human Studies**

**Subjects.** The study included seven healthy, lean, normal glucose-tolerant individuals [age = 37 ± 5 yr; body mass index = 27.1 ± 1.2; fasting plasma glucose = 92 ± 3 mg/dl; 2 h PG during oral glucose tolerance test (OGTT) = 112 ± 9 mg/dl; fasting plasma insulin = 3 ± 1 μU/ml; 2 h plasma insulin = 25 ± 8 μU/ml; fasting plasma FFA = 0.51 ± 0.09 mM; 2 h FFA during OGTT = 0.08 ± 0.007 mM]. All subjects had normal liver, cardiopulmonary, and kidney function as determined by medical history, physical examination, screening blood tests, electrocardiogram, and urinalysis. No normal glucose tolerance subject was taking any medication known to affect glucose tolerance, and no subject participated in a regular exercise program. Body weight was stable (± 2 kg) for at least 3 mo before study in all subjects. The study protocol was approved by the Institutional Review Board of the University of Texas Health Science Center, San Antonio, and informed written consent was obtained from all subjects before their participation. All studies were performed at the General Clinical Research Center (GCRC) of the University of Texas Health Science Center at 0800 following a 10- to 12-h overnight fast.

**OGTT.** Before the OGTT, a catheter was placed in an antecubital vein, and blood samples were collected at −30, −15, 0, 30, 60, 90, and 120 min for measurement of plasma glucose, insulin, and FFA concentrations.

**Muscle biopsies.** On a separate day, subjects returned to GCRC at 0800 following a 10- to 12-h overnight fast for a muscle biopsy. Vastus lateralis muscle samples (~300 mg) were obtained under local anesthesia (1% lidocaine) with a percutaneous needle as previously described (12). Muscle biopsy was placed in buffer on ice for mitochondrial isolation and measurement of mitochondrial ATP production.

**Analytic Techniques**

Plasma glucose was measured by the glucose oxidase reaction (Glucose Oxidase Analyzer; Beckman, Fullerton, CA). Plasma insulin concentration was measured by a radioimmunoassay (Coat A Coat; Diagnostic products, Los Angeles, CA). Plasma FFA was measured by an enzymatic colorimetric method (Wako Chemicals, Neuss, Germany).

**Mitochondrial purification.** Mitochondria (predominately subsarcolemmal) were purified from human or mouse skeletal muscle using methods in long-standing use in the investigator’s laboratory (34). Human tissue (~300 mg) was obtained by biopsy of the vastus lateralis muscle, and mouse tissue was obtained from the back limb skeletal muscle (predominantly gastrocnemius and quadriceps), according to Chappell and Perry (10). Muscle tissue was excised, weighed, and immediately bathed in iced 150 mM KCl solution and then placed in Chappell-Perry buffer. The minced skeletal muscle was homogenized with an all glass homogenizer. The homogenate was centrifuged for 10 min at 600 g, and the supernatant was passed through two cheese cloth layers and centrifuged at 14,000 g for 10 min. The resultant pellet was washed once in modified Chappell-Perry buffer with 0.5% BSA and two times in modified Chappell-Perry buffer without BSA. Mitochondrial integrity was assessed by respiratory control ratio (>6 with pyruvate) at the end of each experiment. All procedures were performed on ice, and the entire isolation procedure lasted ~60–70 min. The final mitochondrial solution was kept on ice and was used immediately following isolation.

**Mitochondrial ATP Production.** Aliquots of the final mitochondrial suspension were used for measurement of mitochondrial ATP production rate with a bioluminescent technique. The reaction mixture included a luciferin-luciferase ATP-monitoring reagent (Roche), substrates for oxidation, and 75 μM ADP. Substrates added were as follows: 5 mM pyruvate plus 5 mM malate, 10 mM succinate plus 0.001 mM rotenone. Fatty acid metabolites [palmitoyl-l-carnitine (PC), palmitoyl-CoA, and oleoyl-CoA] were added as indicated. l-Carnitine (2 mM) was added to the reaction mixture including the blank well, except when the effect of carnitine was tested. Initially, mitochondrial aliquots were incubated at 37°C for 5 min in buffer containing (in mM) 124 KCl, 5 MgCl2, 2 K2HPO4, and 10 HEPES at pH 7.44. Substrates were then added, and the reaction was started with the addition of luciferin-luciferase with ADP. The reaction was performed in a 96-well plate, and plate readings were made with Fluoroscan Ascent instrument (Thermo). A blank well (mitochondria without substrate) was used to measure background, and its value was subtracted from all other wells. All reactions for a given sample were monitored simultaneously and calibrated with addition of an ATP standard (Roche).

**Oxygen uptake measurement.** Oxygen consumption was measured with a Clark-type electrode (Hansatech Instruments, Norfolk, UK) in 0.5 ml of reaction medium (37°C) in a sealed glass cuvette equipped with a magnetic stirrer. The reaction medium used to measure oxygen consumption consisted of 10 mM HEPES buffer, pH 7.44, 125 mM KCl, 5 mM MgCl2, and 2 mM KH2PO4.

**Measurement of complex I–III activity.** Complex III activity was measured as the rate of cytochrome c reduction by the mitochondria in the presence of complex IV inhibitor KCN. Cytochrome c reduction was measured by the change in light absorbance at 550 nm. The reaction was performed in 1-ml cuvettes. The mitochondria were incubated with cytochrome c (5 mM) and KCN (5 mM) until a stable baseline was achieved. The reaction was started by adding the substrate, succinate (10 mM) or pyruvate/malate (5 mM).

**Measurement of inner mitochondrial membrane potential.** Changes across the inner mitochondrial membrane potential were measured by observing safranin O fluorescence using a Fluoroscan plate reader (Thermo) at excitation/emission wavelengths of 530/590 nm. An increase in the fluorescence corresponds to a decrease in the membrane potential. The increase in fluorescence following treatment of the mitochondria with 1 μM carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP) was considered to represent a 100% decrease in membrane potential.
Statistical Analysis

Data are presented as means ± SE. For comparison between groups, Student’s unpaired t-test was used. Statistical significance was considered at \( P < 0.05 \).

RESULTS

Mitochondrial ATP Synthesis with FFA Metabolites in Mouse

Figure 1A shows that, when PC is used as a substrate for ATP synthesis in mitochondria isolated from skeletal muscle of a normal mouse, the relationship between ATP synthesis rate and substrate concentration is biphasic. As PC concentration increases from 0.5 to 2 \( \mu \text{M} \), there is a 42% \(( P < 0.05 \)) increase in the ATP synthesis rate. Antimycin A, a selective inhibitor for mitochondrial complex III, completely inhibited ATP synthesis with 2 \( \mu \text{M} \) PC. Further increases in PC concentration had a deleterious effect on ATP synthesis. As PC concentration increased from 2 to 10 \( \mu \text{M} \), the ATP synthesis rate declined by 65% \(( P = 0.03 \)) below its maximal value. Thus the dose-response curve relating ATP synthesis to PC concentration has an inverted U shape (Fig. 1A). The dose-response curve relating ATP synthesis rate and both palmitoyl-CoA and oleoyl-CoA was very similar to that for PC (Fig. 1A). However, unlike PC, palmitoyl-CoA and oleoyl-CoA supported mitochondrial ATP synthesis only when 2 mM L-carnitine was added to the reaction mixture.

Effect of FFA Metabolites on Mitochondrial ATP Synthesis with Succinate and Pyruvate

To investigate the effect of elevated FFA metabolite concentration on glucose oxidation, we tested the effect of 10 \( \mu \text{M} \) FFA, MITOCHONDRIAL DYSFUNCTION, AND INSULIN RESISTANCE

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Fig. 1. Effect of free fatty acid (FFA) metabolites on ATP synthesis in mitochondria isolated from mouse skeletal muscle. A: full dose-response curve for mitochondrial ATP synthesis with palmitoyl carnitine (PC), palmitoyl-CoA (Pal CoA), and oleoyl-CoA (Ol CoA) as substrates. Carnitine (2 mM) was added to the reaction solution. Data are presented as means ± SE \(( n = 8 \)) . B: % reduction in the mitochondrial ATP synthesis rate with 5 mM pyruvate caused by 10 \( \mu \text{M} \) of various FFA metabolites \(( n = 8 \) for PC and \( n = 5 \) for other metabolites). Car, carnitine; carnitine concentration is 2 mM. C: % reduction in the mitochondrial ATP synthesis rate with 5 mM succinate when 10 \( \mu \text{M} \) of various FFA metabolites are added \(( n = 6 \) for PC and \( n = 5 \) for other metabolites). D: dose-response curve for mitochondrial ATP synthesis rate with pyruvate in the presence of various PC concentrations. E: relationship between the kinetic parameters derived from the curves in D and PC concentration. F: dose-response curve for mitochondrial ATP synthesis with PC as a substrate in skeletal muscle mitochondria isolated from lean healthy subjects. Data are means ± SE \(( n = 7 \) ). G: % reduction in mitochondrial ATP synthesis with 5 mM pyruvate or succinate + rotenone with the addition of 10 \( \mu \text{M} \) PC \(( n = 7 \) ). Values are means ± SE. *\( P < 0.0001 \) and **\( P < 0.01 \) compared with control.
PC on mitochondrial ATP synthesis with pyruvate. Figure 1B shows that 10 μM PC inhibited pyruvate/malate-stimulated ATP synthesis rate and succinate-stimulated ATP synthesis rate (Fig. 1C) by >90% (P < 0.0001). Similarly, 10 μM palmitoyl-CoA and oleoyl-CoA inhibited mitochondrial ATP synthesis with pyruvate and succinate (Fig. 1, B and C). Furthermore, the inhibitory action of both palmitoyl-CoA and oleoyl-CoA was independent of carnitine. l-Carnitine itself (2 mM) or CoA (10 μM) did not affect ATP synthesis with either pyruvate or succinate.

The inhibitory effect of PC on ATP synthesis with pyruvate or succinate was reversible. There was complete recovery of ATP synthesis rate when PC was washed out (Fig. 1, B and C). The inhibitory effect of PC on mitochondrial ATP synthesis with pyruvate also was observed at submaximal concentrations of pyruvate (Fig. 1D). PC markedly decreased the maximal velocity for ATP synthesis with pyruvate (Fig. 1E) without a significant effect on the Michaelis constant. These results indicate that elevated levels of fatty acid metabolites (FA-CoA, FA-carnitine) inhibit mitochondrial substrate oxidation.

**Effect of PC on Human Mitochondrial ATP Synthesis**

To examine whether a similar inhibitory effect of PC on mitochondrial ATP synthesis exists in humans, we performed vastus lateralis muscle biopsies on seven healthy individuals.

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**Effect of PC on Human Mitochondrial ATP Synthesis**

To examine whether a similar inhibitory effect of PC on mitochondrial ATP synthesis exists in humans, we performed vastus lateralis muscle biopsies on seven healthy individuals. Figure 1E shows that, in mitochondria isolated from skeletal muscle of normal healthy subjects, the shape of the dose-response curve relating ATP synthesis to the PC concentration is similar to that of mouse mitochondria and has an inverted U shape (Fig. 1E). Furthermore, 10 μM PC inhibited both pyruvate-stimulated ATP synthesis rate (Fig. 1F) and succinate-stimulated ATP synthesis rate (Fig. 1F) by 89 ± 8 and 89 ± 4, respectively (P < 0.0001), in human muscle mitochondrial like it did in mitochondria isolated from mouse.

**Effect of PC on mitochondrial oxygen consumption.** The inhibitory effect of FFA metabolites on ATP synthesis rate could be due to: 1) inhibition of ATP synthesis by complex V, and 2) uncoupling of electron transport and ATP synthesis. To elucidate the mechanism by which FFA metabolites inhibit ATP synthesis, we examined the effect of PC on oxygen consumption in isolated mouse mitochondria. PC inhibited state 3 respiration in a dose-dependent manner. PC (10 μM) inhibited state 3 respiration with pyruvate by 34% (n = 5; P < 0.05; Fig. 2A), and further increases in PC concentration to 25 μM progressively inhibited oxygen consumption by mouse mitochondria respiring with pyruvate. The inhibitory effect of PC on oxygen consumption was blocked completely by inclusion of 0.1% BSA (binds to PC) in the reaction buffer (data not shown). Furthermore, the inhibitory effect of PC on oxygen consumption also was observed in the presence of the mitochondrial uncoupler FCCP (1 μM) (Fig. 2B). These results suggest that PC inhibits oxygen consumption in mitochondria respiring with pyruvate by interfering with the electron transport chain. Figure 2B shows that PC also inhibits oxygen consumption in mitochondria respiring with succinate in the absence and presence of FCCP. This indicates that the electron transport site, which is inhibited by PC, is distal to complex 1.

**Effect of PC on activity of mitochondrial complexes.** Figure 3A shows that, in the presence of pyruvate as a substrate (Fig. 3A, left), muscle mitochondria strongly reduce cytochrome c. Addition of 10 μM PC to the reaction mixture inhibited the reduction of oxidized cytochrome c by mitochondria, indicating that PC inhibits complex I–III activity. Similarly, 10 μM PC inhibited cytochrome c reduction by mouse skeletal muscle mitochondria when succinate was used as substrate, suggesting that PC also inhibits complex II–III activity. The inhibitory effect of PC on the complex activity was dose dependent (Fig. 3C) and blocked by 0.1% BSA (Fig. 3B, right). These results strongly suggest that PC inhibits the electron transport chain activity.

**Effect of PC on inner mitochondrial membrane potential.** Mitochondrial ATP synthesis is dependent on the electrical potential across the inner mitochondrial membrane, which provides the electromotive driving force for ATP synthesis. The inner mitochondrial membrane potential is completely dependent on the activity of the electron transport chain.
Because PC inhibits the electron transport chain, we postulated that it also might depolarize the inner mitochondrial membrane potential. Figure 4A demonstrates that 10 μM PC reduced the membrane potential across the inner mitochondrial membrane by 40% and that the inhibitory effect of PC on the inner membrane potential is dose dependent (Fig. 4B).

**DISCUSSION**

We have assessed the effect of an elevation in fatty acid metabolite (FACoA and FA carnitine) concentration on ATP synthesis rate in mitochondria isolated from both human and mouse skeletal muscle. FFAs are the principal fuel source for skeletal muscle, and FFA uptake by the mitochondria is the rate-limiting step in FFA oxidation (9). FFAs must be conjugated to CoA and then to carnitine before they can be taken up by the mitochondria (9). In this study, we demonstrate that an increase in fatty acid metabolite concentration exerts a deleterious effect on muscle mitochondrial ATP synthesis. When PC concentration was elevated >2 μM, in mitochondria isolated from mouse skeletal muscle, ATP synthesis precipitously declined. A similar inverted U shape dose-response curve relating mitochondrial ATP synthesis rate and palmitoyl-CoA concentration and oleoyl-CoA concentration was observed, indicating that the deleterious action of elevated fatty acid metabolites is not selective to PC but also extends to other FFA metabolites, e.g., FACoA inhibit ATP synthesis.

FFA metabolites inhibited ATP synthesis with pyruvate by ~90%, indicating that elevated FFA metabolite concentration also inhibits glucose oxidation in mouse skeletal muscle mitochondria. The deleterious effects of FFA metabolites on mitochondrial ATP synthesis also was observed in skeletal muscle mitochondria isolated from healthy normal-glucose-tolerant subjects. These results demonstrate that elevated concentrations of FFA metabolites cause an acquired mitochondrial defect that leads to impaired substrate oxidation in skeletal muscle. Randle (39a) suggested that increased FFA availability/FFA oxidation restrained glucose oxidation by inhibiting pyruvate dehydrogenase. In the present study, we provide Fig. 3. Effect of PC on mitochondrial complex activity. A: change in light absorbance at 550 nm for mitochondria incubated with cytochrome c (5 mM) and KCN (5 mM). Arrows indicate the time at which substances were added. B: change in light absorbance at 550 nm for mitochondria incubated with cytochrome c (5 mM) and KCN (5 mM) (left) and cytochrome c (5 mM), KCN (5 mM), and 0.1% BSA (right). Arrows indicate the time at which substances were added; PC concentration was 10 μM. C: dose-response curve for inhibition of complex II–III activity by PC.

Because PC inhibits the electron transport chain, we postulated that it also might depolarize the inner mitochondrial membrane potential. Figure 4A demonstrates that 10 μM PC reduced the membrane potential across the inner mitochondrial membrane by 40% and that the inhibitory effect of PC on the inner membrane potential is dose dependent (Fig. 4B).

**Fig. 4.** Effect of PC on inner mitochondrial membrane potential. A: change in fluorescence for mitochondria incubated with safranin O (5 μM) and pyruvate (10 mM). Arrow indicates the time at which PC (10 μM) was added to the reaction mixture. B: dose-response curve for the decrease in inner mitochondrial membrane potential by PC. *P < 0.05, **P < 0.001, and ***P < 0.0001.
Based upon the following observations, our results indicate that PC inhibits mitochondrial ATP synthesis by blocking the electron transport chain. 1) PC inhibited oxygen consumption in isolated mitochondria, and this inhibition was observed in the presence of the mitochondrial uncoupler FCCP. The inhibition of oxygen consumption with PC in the presence of the mitochondrial uncoupler strongly indicates that PC directly inhibits the electron transport chain. 2) PC inhibited cytochrome c reduction by skeletal muscle mitochondria. Collectively these results strongly support an inhibitory effect of PC on the electron transport chain. BSA itself did not affect the reduction of cytochrome c by the mitochondria. Thus its ability to prevent the inhibitory action of PC on complex activity most likely is due to its ability to bind to PC, thereby reducing the free PC concentration in the reaction mixture. This observation further supports the conclusion that elevated concentrations of fatty acid metabolites inhibit electron transport chain activity. In the present study, muscle tissue was obtained from lean, healthy, normal-glucose-tolerant subjects. Whether the effect of long-chain FACoAs and FA carnitines on ATP synthesis by mitochondria isolated from muscle of diabetic and obese subjects, the most commonly encountered states of insulin resistance, is similar to that of muscle mitochondria isolated from lean nondiabetic subjects remains to be determined. Of note, low PC concentrations (<5 μM) increased cytochrome c reduction with succinate as a substrate (Fig. 3C). This stimulatory effect of low PC concentrations on complex III activity most likely is explained by the observation that PC, at these low concentrations, is a mitochondrial substrate that contributes electrons through complex I (Fig. 1A), increases the reduced ubiquinone pool, and increases complex III activity.

FACoAs without carnitine cannot be taken up by the mitochondria and, therefore, did not support ATP synthesis. However, they did inhibit mitochondrial ATP synthesis similar to PC. These results indicate that transport of fatty acid metabolites in the mitochondria is not required for their deleterious effect on mitochondrial ATP synthesis.

Our observations provide a mechanism to explain the results of a previous study in which impaired electron transport chain activity was demonstrated in muscle mitochondria isolated from obese and type 2 diabetic insulin-resistant individuals (43). Thus pyruvate donates electrons to complex I, whereas succinate donates electrons to complex II. Because PC inhibited electron transport in mitochondria using succinate or pyruvate as a substrate, it is likely that PC inhibits complex III activity. However, inhibition of complex I and II by PC cannot be excluded. Of note, O center for complex III faces the outside of the inner mitochondrial membrane (34), thus inhibiting its activity, which would not require the transport of FFA metabolites in the mitochondria.

Our results indicate that the inhibitory action of PC on mitochondrial ATP synthesis does not require the inhibition of pyruvate dehydrogenase. Thus ATP synthesis with glutamate was inhibited by the addition of PC, an effect that cannot be explained by the inhibition of pyruvate dehydrogenase activity. Furthermore, palmitoyl-CoA and oleyl-CoA, which are not taken up by the mitochondria, still inhibit ATP synthesis with pyruvate. Therefore, inhibition of pyruvate dehydrogenase by PC is unlikely to explain our observation that FFA metabolites inhibit ATP synthesis with pyruvate.

The inner mitochondrial membrane potential is generated by the electron transport chain and provides the driving force for mitochondrial ATP synthesis. PC reduced the inner mitochondrial potential in a dose-dependent manner. This reduction in inner mitochondrial membrane potential is consistent with the inhibitory action of PC on electron transport chain and contributes to the inhibition of ATP synthesis by PC. Uncoupling of the inner mitochondrial membrane with FFA metabolites also could contribute to the reduction in inner mitochondrial membrane potential.

It is noteworthy that the PC concentration required to obtain complete inhibition of oxygen consumption and complete dissipation of the inner mitochondrial membrane potential is greater than that which caused near complete inhibition of ATP synthesis. One potential explanation for this finding is that the ~40% decrease in inner membrane potential observed with 10 μM PC is sufficient to completely abolish ATP synthesis but not to completely inhibit oxygen consumption. Another possibility is that other deleterious actions of PC on mitochondrial function, e.g., uncoupling of the inner mitochondrial membrane (7) or inhibition of the adenine nucleotide translocase which has been reported in mitochondria isolated from liver and heart (11, 46), also may contribute to the inhibitory action of fatty acid metabolites on ATP synthesis. Such actions of fatty acid metabolites, in the presence of blockade of the electron transport chain, would inhibit ATP synthesis without affecting oxygen consumption by the mitochondria.

Cytosolic FACoAs are intermediates in lipid synthesis/oxidation and primarily are derived from circulating FFAs or intramuscular lipid sources such as triglycerides. The normal plasma FFA concentration is in the range of 350–500 μM. However, the majority (98–99%) of plasma FFAs are bound to albumin. Thus the plasma concentration of FFAs is in the low micromolar range. The intracellular concentration of FACoA varies between tissues and also depends on the nutritional status. In human skeletal muscle, a value of 1–10 nmol/g wet muscle tissue has been reported (2), and similar values have been reported in rats (2, 15, 41). Such a FACoA content would yield an intramyocellular concentration of at least 1–10 μM within the range of concentrations used in the present experiments. Furthermore, whole body insulin sensitivity displays a strong negative correlation with muscle FACoA content such that an increase in FACoA content >2 nmol/g wet muscle tissue (which is equivalent to 2 μM) is associated with a marked reduction in whole body insulin sensitivity (2). In experimental animals, the intracellular FACoAs concentration has been shown to vary among tissues and to be dependent on the nutritional status of the animal. In fasting rats, the basal intracellular FACoA concentration in skeletal muscle can reach as high as 12.5 μM (41). Furthermore, in normal rats, a 16-h fasting caused a 26% increase in mitochondrial FACoA content in association with a 24% reduction in mitochondrial capacity to oxidize fat (32). Thus the deleterious effects of fatty acid metabolites observed in the present study are well within the physiological range of intracellular fatty acyl-CoAs. Moreover, physiological manipulations, e.g., prolonged fasting, which increase intramitochondrial FACoA concentrations to levels observed in the present study result in impaired mitochondrial substrate oxidation. Although in vitro studies with
rodent liver suggest that the intracellular concentration of free FFA carnitine may be less than 1 μM due to the presence of binding proteins (16), it has yet to be demonstrated that such binding proteins exist in human skeletal muscle in vivo. Moreover, intracellular compartmentalization could result in FFA carnitine concentrations even higher than those used in the present study.

Studies with magnetic resonance spectroscopy (MRS) have reported a mitochondrial defect in oxidative phosphorylation in insulin-resistant individuals (38), and a similar defect can be produced by elevating the plasma FFA concentration in lean, healthy, insulin-sensitive individuals (9). A mitochondrial defect measured in vivo with MRS could result from a decrease in mitochondrial density with normal mitochondrial function or from a functional mitochondrial defect in oxidative phosphorylation. The mitochondrial defect observed following acute elevation (6 h) in plasma FFA concentration most likely results from an acquired functional defect rather than from a decrease in mitochondrial number (9, 44). Our in vitro results are consistent with this interpretation by demonstrating a direct inhibitory effect of fatty acid metabolites on mitochondrial oxidative phosphorylation. Our results also are consistent with previous studies that have reported a functional defect in the electron transport chain in obese nondiabetic and type 2 diabetic subjects (43).

ATP synthesis rate normalized per milligram mitochondrial protein was two to three times higher in human skeletal muscle compared with mouse skeletal muscle. This suggests that oxidative phosphorylation in human skeletal muscle mitochondria is more efficient than in mouse. Although the mouse has a higher basal metabolic rate than humans, most of the increased metabolic rate in the mouse is directed toward heat production to maintain body temperature. Thus lower efficiency in ATP production is consistent with less coupled mitochondria and increased heat production in mouse mitochondria compared with humans. It also should be noted that in vivo measurement of the ATP/Oxygen (P/O) ratio in humans has revealed greater values compared with rodents (1, 30).

The ATP synthesis rate measured in human skeletal muscle in vivo ranges between 2 and 10 μmol/g muscle−1·min−1 (9, 38), and this is only slightly lower than the maximal ATP synthesis rate (~20 μmol/g protein) observed in the present study. However, it should be noted that our in vitro measurement is normalized per gram mitochondrial protein, whereas the in vivo measurement is normalized per gram muscle weight. Furthermore, our in vitro measurement was made with maximal substrate concentration, whereas the in vivo measurement was obtained under submaximal conditions.

An elevation in the intramyocellular FFA carnitine concentration has been implicated in the pathogenesis of insulin resistance. An increase in intramyocellular FFA carnitine concentration in rodents fed a high-fat diet (9) or overexpression of lipoprotein lipase in the skeletal muscle to increase the intramuscular FFA levels (24) has been shown to result in insulin resistance. Conversely, depletion of intramyocellular FFA carnitine levels by weight loss in morbidly obese subjects (24), or by reducing the plasma FFA concentration with acipimox (3) in subjects with type 2 diabetes, enhances insulin action. The results of the present study provide strong evidence for an acquired mitochondrial defect that is caused by a physiological increase in intracellular FFA carnitine concentration in skeletal muscle. We therefore postulate that a rise in intramyocellular FFA carnitine interferes with mitochondrial ATP synthesis by inhibiting the electron transport chain and decreasing the inner mitochondrial membrane potential. As a result, FFA carnitine oxidation is reduced, leading to a further rise in intracellular FFA carnitine concentration and exacerbation of the mitochondrial dysfunction. This sequence of events leads to a self-perpetuating negative feedback cycle whereby a small rise in intramyocellular FFA carnitine impairs mitochondrial function and further increases the intramyocellular FFA carnitine concentration. Measurement of mitochondrial ATP synthesis rate in subjects with and without insulin resistance would be required to confirm this hypothesis.

In summary, we have shown that a FFA carnitine concentration >2 μM inhibits ATP synthesis in isolated mitochondria from skeletal muscle of mice and normal healthy individuals. This deleterious action of FFA metabolites on mitochondrial substrate oxidation provides a potential mechanism that can link lipotoxicity, mitochondrial dysfunction, and insulin resistance.

REFERENCES


21. MacDonald KG, Shulman GI.

22. MacDonald KG, Shulman GI.

23. McGarry JD.


