Mitochondrial Protein Acylation and Intermediary Metabolism: Regulation by Sirtuins and Implications for Metabolic Disease*

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This work was supported, in whole or in part, by National Institutes of Health grants P30 DK026743 (to the UCSF Liver Center) and R24 DK085610 from NIDDK. This work was also supported by a Senior Scholarship in Aging from the Ellison Medical Foundation and institutional support from the J. David Gladstone Institutes. Eric Verdin is a member of the Scientific Advisory Board of Sirtris/GSK, a company involved in the commercialization of sirtuin-related discoveries. This is the third article in the Thematic Minireview Series on Sirtuins: From Biochemistry to Health and Disease.

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The sirtuins are a family of NAD+-dependent protein deacetylases that regulate cell survival, metabolism, and longevity. Three sirtuins, SIRT3–5, localize to mitochondria. Expression of SIRT3 is selectively activated during fasting and calorie restriction. SIRT3 regulates the acetylation level and enzymatic activity of key metabolic enzymes, such as acetyl-CoA synthetase, long-chain acyl-CoA dehydrogenase, and 3-hydroxy-3-methylglutaryl-CoA synthase 2, and enhances fat metabolism during fasting. SIRT5 exhibits demalonylase/desuccinylase activity, and lysine succinylation and malonylation are abundant mitochondrial protein modifications. No convincing enzymatic activity has been reported for SIRT4. Here, we review the emerging role of mitochondrial sirtuins as metabolic sensors that respond to changes in the energy status of the cell and modulate the activities of key metabolic enzymes via protein deacylation.

Proper mitochondrial function is required for metabolic homeostasis and involves careful regulation of the activity of multiple metabolic enzymes. Changes in mitochondrial number and activity are implicated in aging, cancer, and the pathogenesis of the metabolic syndrome, a group of metabolic abnormalities characterized by central obesity, dyslipidemia, high blood pressure, and increased fasting glucose levels (1).

Protein acetylation is increasingly recognized as an important post-translational modification for a number of key mitochondrial pathways (2, 3). Lysine malonylation and succinylation were recently identified in several mitochondrial proteins, and the mitochondrial sirtuin SIRT5 was found to have demalonylase/desuccinylase activity. Here, we review the emerging role of protein acylation and its regulation by sirtuins in mitochondrial biology and metabolic regulation.

Three Mitochondrial Sirtuins

Mammals contain seven sirtuins (SIRT1–7) that are characterized by an evolutionarily conserved sirtuin core domain homologous to Sir2, a yeast protein that increases life span (4, 5). SIRT1–7 are localized in distinct subcellular compartments. SIRT1, SIRT6, and SIRT7 are found in the nucleus; SIRT2 is primarily cytosolic; and SIRT3–5 are found in mitochondria. Sirtuins have different levels of NAD+-dependent protein deacetylase activity. This reaction couples lysine deacetylation to NAD⁺ hydrolysis to yield O-acetyl-ADP-ribose, the deacetylated substrate, and nicotinamide (reviewed in Refs. 6 and 7). SIRT1–3 exhibit robust protein deacetylase activity, whereas the others have only weak and highly selective (SIRT5–7) or undetectable (SIRT4) protein deacetylase activity. So far, only weak ADP-ribosyltransferase activity has been described for SIRT4 (8, 9). The dependence of sirtuins on NAD⁺ suggests that their enzymatic activity is directly linked to the energy status of the cell via the cellular NAD⁺:NADH ratio; the absolute levels of NAD⁺, NADH, or nicotinamide; or a combination of these variables (10–14).

Mitochondrial Protein Acetylation

Reversible protein acetylation occurs primarily at the ε-amino group of lysine residues (for a recent review of mechanistically distinct N-terminal acetylation, see Ref. 15). Like other post-translational modifications, lysine acetylation regulates diverse protein properties, including DNA-protein interactions, subcellular localization, protein stability, protein–protein interaction, and enzymatic activity (16).

Mitochondrial proteins are subject to extensive lysine acetylation (17, 18). Acetylated mitochondrial proteins include those involved in energy metabolism, such as in the TCA cycle, oxidative phosphorylation, β-oxidation of lipids, amino acid metabolism, carbohydrate metabolism, nucleotide metabolism, and the urea cycle (2, 3). Interestingly, 44% of mitochondrial dehydrogenases are acetylated. Among them, 14 use NAD⁺ as the electron acceptor to catalyze biochemical reactions in oxidative catalytic routes. The importance of acetylation is further supported by the high degree of conservation of many sites from Drosophila to humans (19).

SIRT3 Is the Major Mitochondrial Protein Deacetylase

Endogenous SIRT3 is a soluble protein in the mitochondrial matrix (20, 21). Interestingly, SIRT3 is translated in the cytoplasm as a longer, enzymatically inactive precursor and imported into the mitochondrion. After import, the first 100 amino acids of SIRT3 are proteolytically cleaved, leading to a final enzymatically active SIRT3 of 28 kDa. A small fraction of SIRT3 resides in the nucleus as well (22). The initial controversy regarding the mitochondrial localization of mouse SIRT3 was resolved by cloning of additional mouse SIRT3 cDNAs that encode a protein that is imported to the mitochondrial matrix, like human SIRT3 (23, 24).

SIRT3 appears to be the major mitochondrial deacetylase because mice lacking SIRT3, but not mice lacking SIRT4 or...
SIRT3, show a striking hyperacetylation of mitochondrial proteins (25). SIRT3 expression is highest in the most metabolically active tissues, including liver, kidney, and heart (26, 27), and is increased in glucose-poor fasting states, including calorie restriction in liver and kidney (28–32). Expression in skeletal muscle also increases under calorie restriction (31, 33) but has been reported to both increase and decrease with fasting (26, 29, 33). Interestingly, SIRT3 expression initially increases on a high-fat diet (HFD) in liver and skeletal muscle, but chronic high-fat feeding leads to decreased SIRT3 expression (26, 31, 33–35). SIRT3 expression also decreases in mouse models of type 2 diabetes mellitus (26, 33).

SIRT3 Regulates Intermediary Metabolism

SIRT3 targets many enzymes that together help mediate the switch to fasting metabolism, as tissues move away from glucose as a source of energy and metabolic intermediates to instead utilize lipids and amino acids.

Lipid Metabolism—SIRT3 promotes the efficient utilization of lipids as a primary source of acetyl-CoA during fasting by deacetylating and activating long-chain acyl-CoA dehydrogenase, a key enzyme in the β-oxidation of fatty acids (28). Mice lacking SIRT3 accumulate β-oxidation precursors and intermediates, including triglycerides and long-chain fatty acids. These mice also share other characteristics of human disorders of fatty acid oxidation, including cold intolerance and reduced basal ATP levels (28). SIRT3 also regulates ketone body production by deacetylating and activating 3-hydroxy-3-methylglutaryl-CoA synthase 2, the rate-limiting enzyme in ketone body biosynthesis. Accordingly, mice lacking SIRT3 show reduced fasting serum levels of ketone bodies (36). SIRT3 also deacetylates and activates acetyl-CoA synthetase 2, an enzyme in extrahepatic tissues that activates acetate into acetyl-CoA (21, 37). SIRT3 deficiency results in fat accumulation in mouse models of type 2 diabetes mellitus (26, 33).

Nitrogen Metabolism—Oxidation of acetyl-CoA to CO₂ by the TCA cycle is a central pathway in energy metabolism. However, the TCA cycle also functions in biosynthetic pathways in which intermediates leave the cycle to be converted primarily to glucose, fatty acids, or nonessential amino acids. Equilibrium of the substrates of the TCA cycle is maintained by two processes called anaplerosis and cataplerosis. Anaplerosis refers to the replenishment of critical anions. Pyruvate carboxylase, which generates oxalacetate directly in the mitochondria, is the major anaplerotic enzyme. Conversely, 4- and 5-carbon intermediates that enter the TCA cycle during the catabolism of amino acids cannot be fully oxidized and therefore must be removed by cataplerosis. Cataplerosis may in turn be linked to biosynthetic processes, such as hepatic gluconeogenesis, fatty acid synthesis in the liver, and glyceroenogenesis in adipose tissue. SIRT3 accelerates amino acid catabolism and nitrogen waste disposal by deacetylating and activating GLUD1 (glutamate dehydrogenase 1), a major cataplerotic enzyme (38). Catabolism of most amino acids requires transfer of the α-amino moiety to α-ketoglutarate by an aminotransferase, forming glutamate. GLUD1 regenerates α-ketoglutarate from glutamate and releases nitrogen to the urea cycle as ammonia (39). SIRT3 accelerates the urea cycle by deacetylating and activating ornithine transcarbamylase, the key mitochondrial enzyme in the urea cycle. Mice lacking SIRT3 exhibit a metabolic profile similar to that in human disorders of the urea cycle, including increased serum ornithine and reduced citrulline levels (the substrate and product, respectively, of ornithine transcarbamylase) (30).

Carbohydrate Metabolism—By promoting fat oxidation, SIRT3 indirectly suppresses carbohydrate utilization. In contrast, cancer cells favor glucose as a source of energy, a process referred to as the Warburg effect (40). SIRT3 down-regulation is frequently observed in tumors and enhances glucose utilization by allowing an increase in reactive oxygen species (ROS) that stimulate hypoxia-inducible factor 1α, a transcription factor that drives the expression of glycolytic genes (41–43). SIRT3 also regulates the acetylation of the peptidyl-prolyl isomerase cyclophilin D. In the absence of SIRT3, this leads to activation of hexokinase II on the outer mitochondrial membrane, facilitating the rapid production of glucose 6-phosphate (41, 44).

Reactive Oxygen Species—SIRT3 also regulates the production of ROS generated as a by-product of oxidative phosphorylation. First, SIRT3 deacetylates and activates isocitrate dehydrogenase 2, an enzyme in the TCA cycle that helps to replenish the mitochondrial pool of NADPH (45). NADPH is used by glutathione reductase to maintain glutathione in its reduced antioxidant form. Second, SIRT3 deacetylates and activates the ROS-scavenging enzyme manganese superoxide dismutase, thereby reducing oxidative damage in the liver (46–48). Mice lacking SIRT3 therefore show increased oxidative stress (46), particularly on a HFD (34), and lose the reduction of ROS levels normally observed under calorie restriction (45).

Oxidative Phosphorylation—Mice lacking SIRT3 consume 10% less O₂ and produce up to 50% less ATP than wild-type mice, suggesting that SIRT3 regulates the activity of the respiratory chain (27, 33). SIRT3 deacetylates and activates mitochondrial respiratory chain complexes, including NDUFA9 (complex I) (27) and SDHA (complex II) (43, 49). Accordingly, mice lacking SIRT3 have lower complex I and II activities than wild-type mice (43, 49). SIRT3 also regulates ATP synthase (35).

Accelerated Metabolic Syndrome in the Absence of SIRT3

The metabolic syndrome is defined by central obesity, insulin resistance, hyperlipidemia, hyperglycemia, and hypertension (50). Physical inactivity, diet, and several genes and their products (including leptin, β₃-adrenergic receptor, hormone-sensitive lipase, lipoprotein lipase, insulin receptor substrate 1, PC-1, and skeletal muscle glycogen synthase) are implicated in the pathogenesis of the metabolic syndrome (51–54). Other meta-

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2 The abbreviations used are: HFD, high-fat diet; ROS, reactive oxygen species; PGC-1α, peroxisome proliferator-activated receptor-γ coactivator 1α; CRAT, carnitine acetyltransferase; ACC, acetyl-CoA carboxylase; MCD, malonyl-CoA decarboxylase.
bolic abnormalities, such as aberrant lipogenesis (55, 56), increased inflammation (57, 58), reduced fatty acid oxidation (59, 60), and increased oxidative stress, have also been implicated. Sustained weight loss and exercise are protective, as might be increased activation of fatty acid oxidation (61).

Lack of SIRT3 and the resulting mitochondrial protein hyperacetylation are associated with accelerated development of the metabolic syndrome (34). Wild-type mice fed a HFD develop obesity, hyperlipidemia, type 2 diabetes mellitus, insulin resistance, and non-alcoholic steatohepatitis (62–65). We reported that the development of each of these consequences of HFD feeding is significantly accelerated in mice lacking SIRT3 (34). In addition, mice lacking SIRT3 show dramatically enhanced levels of proinflammatory cytokines, including IL-6 and TNF-α, another frequent manifestation of the metabolic syndrome. Finally, we found that >90% of SIRT3 knock-out mice develop hepatocellular carcinoma, a cancer associated with the metabolic syndrome in humans (66), when placed on a HFD.3

Interestingly, prolonged exposure (>13 weeks) to HFD feeding in wild-type mice results in a reduction of hepatic SIRT3 expression (34, 35), whereas acute HFD feeding leads to a temporary increase in SIRT3 protein expression (34). A HFD suppresses SIRT3 expression via suppression of peroxisome proliferator-activated receptor-γ coactivator 1α (PGC-1α) (67, 68), a major regulator of SIRT3 expression (69).4 Reintroducing exogenous PGC-1α rescues the loss of SIRT3 in HFD-fed mice (34).

Preliminary evidence also supports a role of SIRT3 in the pathogenesis of the metabolic syndrome in humans. In a population characterized by fatty liver disease (The NASH Clinical Research Network), patients meeting the criteria for metabolic syndrome were more likely to carry the SIRT3 rs11246020 “A” minor allele. In a follow-up study of ~8000 Finnish men focusing specifically on rs11246020, the frequency of this allele and a metabolic syndrome diagnosis were significantly correlated (34). However, this association was relatively weak (odds ratio of 1.3) and was not observed with all definitions of the metabolic syndrome. Remarkably, the SIRT3 rs11246020 polymorphism induces a mutation within the catalytic domain of SIRT3 (V208I). Mutation of Val-208 to isoleucine reduces SIRT3 enzyme efficiency by increasing the Km for NAD+ and reducing the Vmax, consistent with the model that reduction of SIRT3 enzymatic activity increases susceptibility to the metabolic syndrome.

SIRT3, Acetylation, and Metabolic Inflexibility

We hypothesize that high mitochondrial acetyl-CoA levels and mitochondrial protein hyperacetylation cause metabolic inflexibility. Acetyl-CoA, malonyl-CoA, and succinyl-CoA are important intracellular metabolites. They are present in mitochondria and the cytosol and are variously derived from the catabolism of carbohydrates, fatty acids, or proteins (Fig. 1). Intramitochondrial concentrations of acetyl-CoA and succinyl-CoA are in the millimolar range (70), a level that can initiate non-enzymatic acetylation reactions (71). Importantly, global protein acetylation in mitochondria correlates with elevated production of acetyl-CoA in such varied states as fasting, calorie restriction, HFD, and ethanol intoxication (28, 34, 72–74).

Acetyl-CoA is produced during the aerobic catabolism of carbohydrates from pyruvate, during β-oxidation of long-chain fatty acids, and from the catabolism of some amino acids or

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3 M. D. Hirschey and E. Verdin, unpublished data.
4 J. Y. Huang and E. Verdin, unpublished data.
decarboxylation of malonyl-CoA (Fig. 1) (39). There is growing evidence that levels of acetyl-CoA regulate fuel utilization and that dysregulated acetyl-CoA levels have a role in the pathogenesis of insulin resistance and the metabolic syndrome. During fasting, acetyl-CoA can either feed into the TCA cycle for energy production or be used for ketogenesis or acetate production (primarily in the liver) (39). During feeding, acetyl-CoA is exported from the mitochondria to the cytoplasm via citrate by the activity of ATP citrate lyase. Excess acetyl-CoA can also be exported from the mitochondria via the activity of the enzyme carnitine acetyltransferase (CrAT) (75). This enzyme is present in the mitochondrial matrix and combines acetyl-CoA and carnitine into acetyl-carnitine (76), which can be directly exchanged across the mitochondrial membrane by carnitine/acetyl-carnitine translocase (Fig. 1).

Increased mitochondrial levels of acetyl-CoA induced by fatty acid oxidation allosterically inhibit the activity of pyruvate dehydrogenase, a mitochondrial enzyme complex that converts pyruvate into acetyl-CoA and thereby couples glycolysis and glucose oxidation. This acetyl-CoA-mediated inhibition represents part of the glucose-fatty acid cycle originally proposed by Randle to explain the lipid-induced suppression of muscle glucose disposal, a hallmark of obesity-associated insulin resistance (77). A pivotal role of intramitochondrial acetyl-CoA concentrations in metabolic control is further supported by recent studies of CrAT (75). Mice with a muscle-specific deletion of CrAT exhibit compromised glucose tolerance and decreased metabolic flexibility. This latter phenomenon was recently identified in obese humans as an inability to switch from fatty acid to glucose oxidation during the transition from fasting to feeding and may be a key manifestation of the metabolic syndrome (78). Muoio et al. (75) proposed that CrAT promotes metabolic flexibility and increases insulin action by enhancing mitochondrial export of excess acetyl residues. On the basis of our observations of conditions associated with high acetyl-CoA levels, we predict that CrAT deletion leads to mitochondrial protein hyperacetylation and that dysregulated mitochondrial protein acetylation might represent the molecular mechanism of metabolic inflexibility. In this context, the ability of SIRT3 to remove excess mitochondrial protein acetylation could therefore lead to increased metabolic flexibility and increased insulin sensitivity.

SIRT4, an Enzyme without a Substrate

Unlike the well defined role of SIRT3 in acetylation, the precise enzymatic function of SIRT4 is unclear. It may possess weak ADP-ribosyltransferase activity (8, 9); however, this activity is >1000-fold slower than that of a bacterial ADP-ribosyltransferase, raising doubt about its physiological significance (79). SIRT4 regulates insulin secretion (8, 9). Intriguingly and unlike SIRT3, SIRT4 expression is reduced during calorie restriction and is increased in mouse models of diabetes (72, 80). SIRT4 negatively regulates fatty acid oxidation in liver and muscle: knockdown of SIRT4 expression enhances fatty acid oxidation and mitochondrial respiration (80). This may be mediated by increased SIRT1, PGC-1α, and CPT1 expression in the absence of SIRT4 (80). Nevertheless, it is not clear how lack of SIRT4 in the mitochondrion affects gene transcription in the nucleus. Identifying the true enzymatic activity of SIRT4 will undoubtedly shed light on its function.

SIRT5, a Protein Demalonylase and Desuccinylase

SIRT5 possesses unique potent demalonylase and desuccinylase activities (79, 81). Malonyllsine and succinyllysine modifications occur in a variety of organisms from yeast to human (81, 82). Malonylation and succinylation are detected in metabolic enzymes, including isocitrate dehydrogenase 2, serine hydroxymethyltransferase, glyceraldehyde-3-phosphate dehydrogenase, GLUD1, malate dehydrogenase 2, citrate synthase, carbamoyl phosphate synthetase 1, 3-hydroxy-3-methylglutaryl-CoA synthase 2, thiosulfate sulfurtransferase, and aspartate aminotransferase (79, 81, 82). Mice lacking SIRT5 show global protein hypermalonylation and hypersuccinylation, suggesting that it is the major protein demalonylase and desuccinylase (81). The biological significance of lysine malonylation and succinylation and how lysine malonylation and succinylation regulate enzymatic activity are currently unknown. In addition to these novel enzymatic activities, SIRT5 may also function as a protein deacetylase on a restricted number of substrates, such as the urea cycle enzyme carbamoyl phosphate synthetase 1 (83).

Succinyl-CoA and Malonyl-CoA Are Critical Metabolic Intermediates

As we discussed above, hyperacetylation of mitochondrial proteins associated with loss of SIRT3 disrupts the normal metabolic switch toward fatty acid utilization that occurs during prolonged fasting (28, 34). Because many metabolic enzymes are also malonylated or succinylated (81, 82), SIRT5-mediated demalonylation or desuccinylation of metabolic enzymes may modulate metabolic pathways in a similar fashion under conditions of high malonyl-CoA or succinyl-CoA levels. SIRT5 is therefore likely to emerge in the future as an important regulator of intermediary metabolism.

Succinyl-CoA is an intermediate in the TCA cycle and also a precursor for porphyrin synthesis (39). Catabolism of odd-chain fatty acids and of some amino acids (e.g. branched-chain amino acids, such as leucine, isoleucine, and valine) generates propionyl-CoA, which is first carboxylated to methylmalonyl-CoA and then converted to succinyl-CoA (Fig. 1) (39). Branched-chain amino acids are the most abundant essential amino acids (84). Muscle represents ~40% of the total mass of mammals and is therefore the largest metabolic organ. Muscle acts as a critical fuel reserve site in starvation or other glucose-poor states (85, 86) and accounts for >50% of the capacity of the tissues to catabolize branched-chain amino acids (87). We therefore expect that succinyl-CoA production will rise during fasting. We do not know yet whether succinyl-CoA levels and global protein succinylation correlate, as is observed between acetyl-CoA levels and mitochondrial protein acetylation.

Malonyl-CoA pools in mitochondria and the cytosol are also tightly regulated. Cytosolic malonyl-CoA is synthesized by the carboxylation of acetyl-CoA by acetyl-CoA carboxylase (ACC), and the decarboxylation of malonyl-CoA by malonyl-CoA decarboxylase (MCD) regenerates acetyl-CoA (88). However, the mitochondrial pool of malonyl-CoA is generated by the
activity of propionyl-CoA carboxylase on acetyl-CoA, with the reverse reaction again catalyzed by MCD (88). ACC and MCD are tightly regulated by a variety of factors, including levels of glucose, insulin, and AMP-activated protein kinase (88). Whole-cell malonyl-CoA levels decrease during fasting and diabetic conditions and rapidly double after feeding (88). Malonyl-CoA is the precursor for de novo fatty acid synthesis but is also a critical inhibitor of fatty acid oxidation. It binds to and inhibits CPT1 on the mitochondrial outer membrane, thereby inhibiting the transport of fatty acids into mitochondria for \( \beta \)-oxidation (88), a regulatory process referred to as the reverse Randle cycle. Drug inhibition or genetic disruption of MCD activity leads to increased intracellular malonyl-CoA levels, decreased fatty acid oxidation, and increased glucose oxidation (89, 90). Mammals encode two isoforms of ACC: ACC1 is enriched in lipogenic tissues, where it produces cytosolic malonyl-CoA as a precursor for lipogenesis, and ACC2 is preferentially expressed in oxidative tissues, where it negatively regulates fatty acid oxidation (91). ACC2 knock-out mice show increased \( \beta \)-oxidation in both liver and muscle. They are lean, hyperphagic, and resistant to obesity and diet-induced diabetes (92, 93). Malonyl-CoA also regulates, directly or indirectly, physiological or pathological conditions, such as muscle contraction, cardiac ischemia, \( \beta \)-cell secretion of insulin, and the hypothalamic control of appetite (88). These findings illustrate the emerging but still partial understanding of the role of malonyl-CoA. The discovery of lysine malonylation as a post-translational modification and its regulation by SIRT5 suggests the intriguing possibility that protein malonylation represents one of the mechanisms by which malonyl-CoA levels regulate intermediary metabolism.

Putting It All Together: Protein Acylation and Regulation of Intermediate Metabolism

The results discussed above support the idea that mitochondrial sirtuins regulate metabolism via the removal of acyl modifications on lysine residues in key enzymes. When nutrient availability changes, the levels of various acyl-CoAs, such as acetyl-CoA, succinyl-CoA, and malonyl-CoA, change correspondingly. The high reactivity of acyl-CoAs, their high mitochondrial concentrations, and the relatively basic pH within the mitochondrial matrix (pH 7.9) all provide conditions favoring non-enzymatic acylation of mitochondrial proteins. Importantly, different nutrients may yield different relative acyl-CoA concentrations as described above for the oxidation of fatty acids versus branched-chain amino acids. The initial function of sirtuins in bacteria during evolution might have been to remove an inadvertent acyl modification on proteins. Such a detoxifying mechanism might have evolved into a complex sensing and regulatory mechanism at a later point, as has been demonstrated for SIRT3.

As discussed above, equilibrium of the substrates of the TCA cycle is maintained by the competing activities of enzymes involved in anaplerosis and cataplerosis. Excess calorie intake, e.g. in the form of a HFD, leads to a relative imbalance with excess anaplerosis and build up of critical intermediate in the TCA cycle, such as acetyl-CoA and succinyl-CoA. This relative increase may in turn lead to increased mitochondrial protein acetylation/succinylation and decreased metabolic flexibility. We propose that SIRT3 and SIRT5, in cooperation with other enzymes in this pathway, such as CrAT, deacytelate and desuccinylate mitochondrial proteins and thereby promote maximal metabolic flexibility. We further propose that a failure of this protective mechanism underlies the pathogenesis of the metabolic syndrome and metabolic inflexibility.

Much work remains to be done to test this mechanism linking mitochondrial protein acylation and metabolic disease. Of particular importance is the identification of the acyl moiety targeted by SIRT4. There are many other acyl-CoAs beside the three discussed in this minireview, all with the potential of inducing the same type of modifications on mitochondrial proteins. Their possible roles in intermediary metabolism and metabolic disease regulation should represent fertile grounds for future investigations. Mechanistic links to other obesity-related diseases, such as diabetic nephropathy, are tempting but remain unknown. Finally, although the ubiquity of protein acetylation argues for a non-enzymatic mechanism, this does not exclude the existence of a specific mitochondrial acetyltransferase. Future research effort should address this important remaining question.

Acknowledgments—We thank John Carroll for figure preparation and Gary Howard for editorial review.

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