S-Adenosyl-L-Methionine Increases Skeletal Muscle Mitochondrial DNA Density and Whole Body Insulin Sensitivity in OLETF Rats

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Abstract
Both mitochondrial dysfunction and alterations in mitochondrial DNA (mtDNA) are implicated in type 2 diabetes mellitus and insulin resistance. Evidence also suggests that metabolism of S-adenosyl-L-methionine (SAM), the universal methyl donor for biological methylation, is associated with mitochondrial dysfunction and insulin resistance. We investigated the effect of SAM on mtDNA density and insulin sensitivity using the Otsuka Long-Evans Tokushima Fatty (OLETF) rat, an animal model of type 2 diabetes mellitus and insulin resistance. To determine the short-term effect on mtDNA density, SAM (15 mg·kg\(^{-1}\)·d\(^{-1}\)) was administered intraperitoneally for 7 d to 6 male, 57-wk-old OLETF rats and 6 Long-Evans Tokushima Otsuka (LETO) rats of the same age as a nondiabetic control. To determine the long-term effect, the same dose of SAM was administered daily to 5 male, 6-wk-old OLETF rats until the age of 25 wk; 7 control OLETF rats received vehicle and 7 LETO rats were untreated. Skeletal muscle mtDNA density was measured by either competitive or multiplex PCR and insulin sensitivity was measured by hyperinsulinemic-euglycemic clamp. SAM treatment for 1 wk increased skeletal muscle mtDNA density of both OLETF and LETO rats. The long-term SAM treatment significantly reduced body weight gain as well as increased skeletal muscle mtDNA density and whole body insulin sensitivity in OLETF rats compared with their vehicle-treated controls. Furthermore, in all 3 groups, skeletal muscle mtDNA density correlated with insulin sensitivity (r = 0.752, P < 0.001). In conclusion, SAM treatment increased mtDNA density in the skeletal muscle, improved whole body insulin sensitivity, and prevented body weight gain in OLETF rats.

Introduction
Mitochondria are intracellular organelles that contain their own DNA encoding 13 proteins involved in oxidative phosphorylation, which is essential for normal fuel metabolism. Qualitative changes in the mitochondrial DNA (mtDNA), such as mutations or deletions, represent a well-known genetic cause of diabetes mellitus (1–3). A quantitative change in the mtDNA is also associated with type 2 diabetes mellitus and insulin resistance (4–8). It was recently reported that insulin resistance in the skeletal muscle of insulin-resistant offspring of patients with type 2 diabetes is attributable to the inherited defect in mitochondrial oxidative phosphorylation (9). An age-associated decline in mitochondrial function also contributes to insulin resistance in the elderly (10). In agreement with these findings, we previously reported that mtDNA density in peripheral blood cells is decreased in type 2 diabetic patients, even before the onset of the disease (4), and in their offspring (8). The mtDNA density measured in peripheral blood cells is inversely related to waist-hip ratio, blood pressure, fasting plasma glucose, and fasting plasma insulin (4,5), whereas it is positively related to the fat oxidation rate (7). A human liver cell line lacking mtDNA was shown to have reduced glucose uptake rate and decreased steady-state mRNA and protein levels of glucose transporters (6). The amount of mtDNA per mitochondrion is generally considered to be constant in all mammalian cell types (11). Taken together, these reports support the notion that mtDNA density is a useful surrogate marker of insulin resistance by reflecting cellular mitochondrial number or mass.

S-Adenosyl-L-methionine (SAM) plays an important role in regulating mitochondrial function (12). It is present in all living cells and organisms and is synthesized from methionine and ATP (13,14). Its known biochemical functions are: 1) a donor of...
methyl groups in many biological methylation reactions (transmethylation pathway); 2) a sulfur-containing metabolite for the transmethyl transferase pathway that leads to the synthesis of cysteine and glutathione; and 3) a precursor molecule for the aminopropylation pathway that provokes the synthesis of polyamines (15,16). The therapeutic application of SAM has mainly been tested in a steatohepatitis model and oral SAM administration was shown to provide protection against liver injury in a dietary model of nonalcoholic steatohepatitis (17). It was reported that mice deficient in hepatic SAM synthesis (homozygous knockout for methionine adenosyltransferase 1A gene), which result in spontaneous development of steatohepatitis, have a decreased mitochondrial function (12). In this mouse model, proteome analysis showed that the decreased mitochondrial function could be explained by a decreased amount of prohibitin, cytochrome c oxidase I and II, and ATPase $\beta$-subunit, which was also observed in ob/ob mice and obese human subjects. SAM regulates mitochondrial function by modulating prohibitin 1 protein level in hepatocytes (12). Therefore, SAM deficiency might result in mitochondrial dysfunction, which might eventually lead to insulin resistance.

A few recent papers suggested that diabetes mellitus is associated with altered SAM metabolism (18–20). It was reported that the blood concentration of SAM was decreased in diabetic patients with kidney dysfunction and also SAM metabolism was disturbed and associated with hyperhomocysteinemia in those patients (19). In a study of insulin-resistant Zucker fatty rats (18), an insulin-sensitizing drug, troglitazone, decreased plasma total homocysteine by affecting the transsulfuration pathway of SAM metabolism.

From these findings, we hypothesized that SAM supplementation could increase insulin sensitivity through the improvement of mitochondrial function. Using a rat model of obese type 2 diabetes mellitus, we examined whether SAM treatment increases mtDNA density, a surrogate of mitochondrial function, and thereby increases insulin sensitivity.

**Materials and Methods**

**Animals, diet, and experimental designs.** All procedures referring to the animal study were approved by the Institutional Animal Care and Use Committee at the Clinical Research Institute of Seoul National University Hospital. Rats were housed in a wire-mesh cage in a temperature- ($21 \pm 2^\circ C$) and humidity-controlled (53%) condition and with a standard 12-h-light/12-h-dark cycle (light on from 0700) at the specific pathogen-free animal facility in the Clinical Research Institute of Seoul National University Hospital. Rats consumed a standard rat diet (Samyang) and tap water ad libitum.

The first study was conducted to determine whether short-term SAM supplementation increases mtDNA density in the skeletal muscles of diabetic Otsuka Long-Evans Tokushima Fatty (OLETF) rats and Long-Evans Tokushima Otsuka (LETO) rats. The OLETF rat, developed by Kawano et al. (21), is a genetic model of spontaneous type 2 diabetes. OLETF rats exhibit hyperglycemic obesity with hyperinsulinemia and insulin resistance similar to that in humans. LETO rats, which originated from the same Long-Evans stock, show normal responses to oral glucose tolerance tests throughout life and have been used as nondiabetic counterparts of OLETF rats (22). SAM-sulfate-p-toluene sulfonate (Aptomil, Yooyoung) dissolved in vehicle (30 g/L l-lysine) was intraperitoneally injected for 7 d (15 mg · kg $^{-1}$ · d $^{-1}$) for 6 male, 57-wk-old OLETF rats and 6 LETO rats of the same age. We chose 57-wk-old rats, because at this age, the diabetic condition is already established or even at an advanced stage. To measure mtDNA density in the skeletal muscle, total DNA was extracted from the vastus lateralis muscle using a QIAamp DNA tissue kit (Qiagen).

The second study was conducted to determine long-term effects of SAM treatment on mtDNA density and insulin resistance as well as subsequent metabolic effects during the development of type 2 diabetes mellitus. Male, 6-wk-old OLETF rats were randomly assigned to receive SAM (15 mg · kg $^{-1}$ · d $^{-1}$, OLETF-SAM, $n = 5$) or vehicle (30 g/L l-lysine, OLETF-control, $n = 7$) daily by intraperitoneal injection until the age of 25 wk. To determine whether SAM treatment reverses the abnormal metabolic status, 7 6-wk-old LETO rats were included as a nondiabetic controls. They were not administered SAM or vehicle. Food intakes for the first 5 wk and body weight throughout the experiment were measured weekly. At 8, 10, and 25 wk of age, rats were killed to obtain tissues for mtDNA quantification.

**Hyperinsulinemic-euglycemic clamp.** The effect of SAM on insulin resistance was measured by the hyperinsulinemic-euglycemic clamp technique (23,24). We placed a catheter into 2 tail veins and a tail artery of each 25-wk-old rat for infusion and blood sampling, respectively. Food was removed at 0800 and the rat was transferred to a quiet, isolated room and weighed. Whole body glucose kinetics was estimated in awake, unstressed rats 6 h after food removal. Rats were allowed to rest for 40 min before we withdrew the first blood sample. Patency of arterial line was maintained by a slow (0.015 mL/min) infusion of saline. A continuous intravenous infusion of purified human insulin (Novolin R; Novo Nordisk) was then started at a rate of 72 pmol · kg $^{-1}$ · min $^{-1}$ and continued for 120 min with a syringe pump (Medfusion 1010i, Medexine) through a tail vein. Insulin was dissolved in 0.9% NaCl containing 0.2% bovine serum albumin (Sigma). Blood samples (50 mL) were taken from the tail artery and immediately centrifuged for glucose measurement at 10-min intervals. Blood samples of 200 mL were collected at 60 and 120 min after insulin infusion for determination of plasma insulin concentrations. Steady-state plasma glucose concentrations were reached after 40–50 min. A 25% dextrose solution was infused through the venous line at variable rates to maintain plasma glucose at baseline levels. After hyperinsulinemic-euglycemic clamp, rats were killed for the measurements of mtDNA density, glutathione, and DNA methylation in quadriceps muscle as well as epidymal fat weight, which indicate visceral obesity.

**Quantification of mtDNA density.** The mtDNA density was measured by either competitive PCR or multiplex PCR. Competitive PCR for mtDNA quantification was performed as previously described (25). The inter-assay variance of mtDNA measurement was 12.2%. For multiplex PCR with nuclear DNA, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), the primers for mtDNA, were the same as for competitive PCR. The sequences of GAPDH primers were: forward, 5'-TCT TCT TGT GCA GTG CCA GC-3' (45–64) and backward, 5'-CTC TCT TGC TCT CAG TAT CC-3' (1101–1120). The PCR reactions were performed at 92°C for 3 min, 60°C for 30 s, and 72°C for 30 s for 1 cycle, and then continued at 94°C for 30 s, 60°C for 30 s, and 72°C for 40 s for the next 23 cycles. The PCR products were analyzed on a 0.8% agarose gel and the band intensity was measured as above. The amount of mtDNA was expressed as a relative value to the GAPDH DNA amount.

**Glutathione concentrations and DNA methylation status in the 25-wk-old rats.** To determine the metabolic effect of SAM administration, the concentration of glutathione, which is an end-product of sulfur-containing amino acid, was analyzed. Quadriceps muscles were rinsed with PBS solution to remove any red blood cells, homogenized in 5–10 mL of cold buffer (50 mmol/L MES, pH 6–7, containing 1 mmol/L EDTA)/g tissue, and then centrifuged at 10,000 × g; 15 min at 4°C. From the supernatant, total glutathione concentrations were measured weekly. At 8, 10, and 25 wk of age, rats were killed to obtain tissues for mtDNA quantification.

**Genomic DNA methylation assay.** To determine the biological methylation effect of administered SAM, genomic DNA methylation was analyzed using liquid chromatography/electrospray ionization mass spectrometry (Hewlett Packard/Bruker). A detailed method description has been previously published (26).
Statistical analysis. Data were expressed as means ± SE. We compared the groups at each time using Student’s t test and Kruskal-Wallis test with post-hoc least significance difference tests. Correlations between variables were determined using Pearson’s correlation analysis. SPSS software version 13.0 was used for all statistical analyses. Differences were considered significant at P < 0.05.

Results

Short-term effect of SAM on mtDNA density. After intraperitoneal injection of SAM for 7 d, the mtDNA densities in the skeletal muscle of 57-wk-old OLETF and LETO rats were increased by 43.7% and 64.7%, respectively (data not shown), which indicates that even a short-term treatment of SAM increases skeletal muscle mtDNA density, but it is not diabetes-specific.

Long-term effect of SAM on food intake, body weight, and insulin sensitivity. Body weights of both OLETF rat groups were higher than those of LETO rats throughout the study (Fig. 1A, Table 1) and long-term SAM treatment significantly reduced weight gain in the OLETF-SAM rats compared with OLETF-controls (Fig. 1A). At 25 wk of age, the body weight of OLETF-SAM rats was about 13% less than that of OLETF-control rats. The relative weight of the bilateral epididymal fat pads of the OLETF-SAM group was significantly lower than that of the OLETF-control group and did not differ from that of LETO rats (Table 1). Both OLETF group rats ate more food than LETO rats and the OLETF-SAM rats ate less food than the OLETF-control group (Fig. 1B).

Plasma glucose and insulin concentrations were measured in food-deprived rats and were significantly higher in both OLETF rat groups compared with LETO rats (Table 1). Both the insulin concentration and the insulin:glucose ratio were significantly lower in the OLETF-SAM group than in the OLETF-control group. During the hyperinsulinemic-euglycemic clamp studies, the glucose infusion rate (GIR) required to maintain euglycemia at the steady state was significantly lower in both OLETF rat groups than in LETO rats. However, the OLETF-SAM group had a higher GIR than the OLETF-control group (Fig. 2).

Effect of SAM on mtDNA density and other parameters in the skeletal muscle. The mtDNA densities of quadriceps muscles were significantly lower in the both OLETF rat groups as compared with those of LETO rats at 8, 10, and 25 wk of age (Fig. 3). Of note, the mtDNA densities in OLETF rats were lower than those in LETO rats even at very young age. However, SAM treatment significantly increased the mtDNA density in OLETF rats at 25 wk of age (Fig. 3) compared with OLETF-control rats.

Considering all rats in the 3 groups, the mtDNA densities in the quadriceps muscles correlated very well with the insulin

### TABLE 1

<table>
<thead>
<tr>
<th></th>
<th>LETO</th>
<th>OLETF-SAM</th>
<th>OLETF-control</th>
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<tbody>
<tr>
<td>n</td>
<td>7</td>
<td>7</td>
<td>5</td>
</tr>
<tr>
<td>Body weight, g</td>
<td>501 ± 12</td>
<td>569 ± 17</td>
<td>657 ± 24</td>
</tr>
<tr>
<td>Epididymal fat pads, % of body weight</td>
<td>1.90 ± 0.16</td>
<td>1.87 ± 0.14</td>
<td>2.42 ± 0.22</td>
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<tr>
<td>Plasma glucose, mmol/L</td>
<td>5.8 ± 0.2</td>
<td>6.6 ± 0.2</td>
<td>6.9 ± 0.5</td>
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<tr>
<td>Plasma insulin, mmol/L</td>
<td>0.38 ± 0.07</td>
<td>0.66 ± 0.09</td>
<td>1.11 ± 0.12</td>
</tr>
<tr>
<td>Insulin:glucose ratio (× 10⁶)</td>
<td>7 ± 1</td>
<td>10 ± 1</td>
<td>16 ± 1</td>
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</table>

1 Values are means ± SE. Means in a row with superscripts without a common letter differ, P < 0.05.

2 Insulin and glucose were measured in plasma obtained from food-deprived rats.
sensitivity index, GIR ($r = 0.752, P < 0.001$; Fig. 4A). Moreover, body weight (LET0<OLETF-SAM<OLETF-control) and mtDNA density in the 3 groups were negatively correlated ($r = -0.696, P = 0.002$; Fig. 4B) as were relative epididymal fat weight and mtDNA density ($r = 0.610, P = 0.009$). The glutathione content and genomic DNA methylation status in the quadriceps muscle did not differ among the 3 groups (data not shown).

**Discussion**

In this study, long-term treatment of SAM during diabetes development resulted in decreased food intake, body weight gain, and visceral adiposity along with improved insulin sensitivity in OLETF rats, a genetic model of type 2 diabetes and insulin resistance. Very importantly, all these changes were significantly associated with mtDNA density in the skeletal muscle.

The mtDNA density in the skeletal muscle of OLETF rats was lower than that of LET0 rats from 8 wk of age. Because OLETF rats develop hyperglycemia around 18 wk of age (21), this observation is consistent with our previous reports that the mtDNA density in peripheral blood cells is decreased in type 2 diabetic patients even before the onset of the disease (4) and in the offspring of diabetic parents (8). The mtDNA density, moreover, is also known to be significantly correlated with insulin sensitivity (5). From these observations, one might speculate that genetically determined quantitative changes in mtDNA are causally related to the development of type 2 diabetes mellitus (27,28) rather than a simple concurrent phenomenon, such as the well-known association between qualitative change in mitochondrial function and diabetogenesis (29). However, no difference was observed in the mtDNA densities of 57-wk-old OLETF and LET0 rats before SAM treatment in this study. Because mtDNA copy numbers decline with aging (30), LET0 rats could also have a low level of mtDNA copies.

In OLETF rats, the lack of body weight gain with SAM treatment coincided with reduced food intake. This observation is consistent with previous studies demonstrating that food restriction reversed obesity and insulin resistance in OLETF rats (31,32). Because OLETF rats have a genetic defect in the cholecystokinin (CCK) receptor A gene, it has been suggested that hyperphagia and obesity are attributable, in this rat model, to the impairment of the CCK-mediated satiety mechanism (33). However, intracerebroventricular administration of SAM in Sprague-Dawley rats also showed an anorexigenic effect (our unpublished data), indicating that the anorexia-inducing effect of SAM is controlled in the central nervous system and not solely attributable to a genetic defect in the CCK receptor A. Because of the lack of a pair-fed group in this study, we are unable to conclude whether the mechanism of an improved insulin sensitivity by SAM treatment was due to a direct stimulating effect on mitochondria or an indirect effect via weight or fat loss associated with decreased appetite.

In this study, the relative weight of the epididymal fat did not differ between the OLETF-SAM group and LET0 group. This is in contrast to a previous report showing that OLETF rats pair-fed with LET0 rats showed more body fat and intra-abdominal fat percentage compared to LET0 rats (34). Thus, we speculate that not only reduced food intake but also increased fat oxidation might contribute to the reduced epididymal fat amount of OLETF-SAM group.

In this study, we found that both long-term SAM treatment for the young OLETF rats and a relatively short period of SAM treatment for the older rats increased the mtDNA density in the quadriceps muscles, which correlated with whole body insulin sensitivity. However, there was no difference in the liver mtDNA density in the OLETF-control rats or OLETF-SAM rats (data not shown). To determine possible mechanisms, we measured the metabolic sequelae of SAM treatment in the muscle. The most plausible candidate mechanism is the effect of SAM against oxidative stress, because SAM can be metabolized to cysteine through the transsulfuration pathway, which is critical in the synthesis of glutathione, a major intracellular antioxidant (15,16). Decreased cellular glutathione content is a common finding in experimental and human diabetes, and glutathione depletion by L-buthionine-[S,R]-sulfoximine, a glutathione synthesis inhibitor, resulted in impaired glucose tolerance (35). Intravenous
glutathione infusion significantly increased total glucose uptake in type 2 diabetic patients (36). In this study, however, skeletal muscle glutathione concentrations did not differ among the groups. It may be speculated that skeletal muscle per se cannot significantly alter the synthesis of glutathione through exogenous SAM, due to a minimal activity of transsulfuration enzymes cystathionine synthase and cystathionase, in such tissue compared to liver, which is instead a major organ for glutathione synthesis (37–39).

Because the availability of SAM is fundamental in biological methylation, we also measured genomic DNA methylation status in the skeletal muscle. We found, however, no difference among these 3 groups, indicating that exogenous SAM does not affect DNA methylation in the skeletal muscle. Because administered SAM increased mtDNA density without changes in genomic DNA methylation, we speculate other possible metabolic effects of SAM on one-carbon metabolism. SAM is an inhibitor of methylenetetrahydrofolate reductase, which irreversibly catalyzes 5, 10 methylenetetrahydrofolate, a coenzymatic form of folate for nucleotide synthesis, to 5-methyltetrahydrofolate, a folate form utilized for biological methylation (39). Thus, inhibition of methylenetetrahydrofolate reductase by SAM can increase the proportion of 5,10 methylenetetrahydrofolate in the folate pool and subsequently increase the availability of nucleotides for DNA synthesis (40), which might provide a condition to synthesize mtDNA efficiently.

In conclusion, long-term treatment of SAM reduced body weight gain and insulin resistance and increased skeletal muscle mtDNA density in OLEFT rats. Furthermore, mtDNA density in the skeletal muscle was correlated with insulin resistance. These observations support the hypothesis that a decrease in mitochondrial mass may be responsible for the development of insulin resistance (9,10,41) and may also provide a clue linking metabolic power of the mitochondrial unit and body mass (42). Further investigation is definitely needed to test whether SAM could be used as an anti-diabetic or an anti-obesity drug.

**Literature Cited**


