Oxidative stress has an important role in the development of obesity and obesity-associated metabolic disorders. As an endogenous antioxidant enzyme, superoxide dismutase 3 (SOD3) has the potential to affect diet-induced obesity and obesity-associated complications. In the current work, we overexpressed SOD3 in C57BL/6 mice fed a high-fat diet (HFD) to study its effect on HFD-induced obesity, fatty liver and insulin resistance. We demonstrated that the Sod3 gene transfer blocked HFD-induced obesity, fatty liver and insulin resistance. Real-time PCR analysis of adipose and liver tissues revealed that overexpression of the Sod3 gene suppressed expression of pro-inflammatory genes in adipose tissue including F4/80, Tnfa, Cdi1lc, Mcp1 and Il6, and increased expression of anti-inflammatory genes such as adiponectin. In the liver, high levels of SOD3 activity in animals enhanced expression of the genes responsible for energy expenditure including Cpt1a, Cpt1b, Pgc1a, Pgc1b and Ucp2. These results suggest that overexpression of the Sod3 gene through gene transfer is an effective approach in preventing diet-induced obesity and obesity-associated complications.
with pLIVE-SEAP plasmids gained about 15 g at the end of an 8-week HFD feeding compared with 5 g in animals injected with pLIVE-SOD3 plasmids. There is no statistical difference in body weights between animals fed a regular chow and those that underwent Sod3 gene transfer. The difference between HFD-fed control and SOD3-treated animals is visually differentiable (Figure 2b). These results suggest that hydrodynamic delivery of pLIVE-SOD3 plasmids completely blocked HFD-induced weight gain. Results from body composition analysis show that the difference between control and SOD3-treated animals is primarily fat mass (Figure 2c) and the lean mass of all animals is not different during the 8-week period (Figure 2d) among the three groups of animals. There is no difference in energy intake among the animals calculated based on the average food intake per animal per day (Figure 2e).

Sod3 gene transfer represses fat and macrophage accumulation in white adipose tissues

Adipose tissues were collected from the Sod3 gene-injected and control animals to study the impact of Sod3 gene transfer. Figure 3a shows the relative amount and size of white and brown adipose tissue in SOD-treated and control animals. Compared with
Sod3 gene transfer blocks HFD-induced obesity in C57BL/6 mice. (a) Time-dependent increase in body weight; (b) photo images of animals fed regular chow (left), HFD with hydrodynamic transfer of pLIVE-SEAP (middle) and HFD with hydrodynamic delivery of pLIVE-SOD3 (right) at the end of 8th week feeding; (c) time-dependent increase of fat mass; (d) time-dependent change of lean mass; and (e) time-dependent energy intake by animals. (●) Animals fed regular chow; (▲) animals fed a HFD and injected with pLIVE-SEAP plasmid; and (▲) animals fed a HFD and injected with pLIVE-SOD3. Data represent mean ± s.e.m. (n = 5). *P < 0.05 compared with Chow mice; #P < 0.05 compared with pLIVE-SEAP-injected animals.

Figure 2.

animals fed a regular chow, the epididymal, inguinal and perirenal white adipose tissues are significantly bigger in size (Figure 3a) and heavier (Figure 3b) in HFD-fed control mice. There is no difference between regular chow-fed mice and HFD-fed mice injected with the Sod3 gene. The average weight of combined white adipose tissues in HFD-fed control animals is 2.1 g, 2.4- and 3.0-fold heavier than that of regular mice and animals that underwent Sod3 gene transfer, respectively. No statistical difference is seen in size or the total weight of brown adipose tissue among the three groups of animals. Images in Figure 3c show the shape and size of adipocytes in white and brown adipose tissue. The average diameter of adipocytes in HFD-fed control animals is 66.6 ± 1.9 μm, compared with animals fed a regular chow (42.7 ± 2.2) or in HFD-fed mice with the Sod3 gene transfer (43.9 ± 2.9; Figure 3d). The crown-like structure, a sign of macrophage infiltration in adipose tissue, is evident in HFD-fed control animals (Figure 3c, insert), but scarce in animals that have undergone Sod3 gene transfer or were fed a regular chow. The relative number of crown-like structures identified in HFD-fed control animals is ~7.3 ± 0.6 mm −2 compared with 1.7 ± 0.3 in animals injected with the Sod3 gene and 2.3 ± 0.2 in those fed a regular chow (Figure 3e). A whistish color of brown adipose tissue sections in HFD-fed control mice suggests that fat content in brown adipose tissue in HFD-fed control animals is higher than that of animals with Sod3 gene overexpression and those fed a regular chow.

Sod3 gene transfer blocks HFD-induced fatty liver

One of the most common physiological changes associated with obesity is an increase in fat content in the liver and the development of fatty liver. Histological images of mouse livers from HFD-fed control mice and animals that received Sod3 gene transfer show significant differences in the number and size of vacuoles in liver sections with H&E staining (Figure 4a). Higher lipid content is indicated in HFD-fed control animals than those of animals injected with the Sod3 gene and regular mice. Results from Oil-red O staining concur with the results of the H&E staining. The average liver weight of HFD-fed control animals is about 2.0 g, compared with ~1.5 g in mice with SOD3 overexpression or regular mice (Figure 4b). Similarly, Sod3 gene transfer also results in normal ranges of triglycerides (Figure 4c) and cholesterol (Figure 4d) and nonesterified fatty acids (Figure 4e) compared with regular mice, and about half that of HFD-fed control mice. These results suggest that Sod3 gene transfer blocked HFD-induced hepatic fat accumulation.

Sod3 gene transfer blocks HFD-induced glucose intolerance and insulin resistance

An intraperitoneal (i.p.) glucose tolerance test and insulin tolerance test were performed to assess the effect of Sod3 gene transfer on animals fed a HFD. Results in Figure 5a suggest that 8-week HFD feeding has caused animals injected with the control plasmid to become glucose intolerant, compared with a normal glucose profile in mice that underwent Sod3 gene transfer, which is identical to that of regular mice. The area under the curve of the intraperitoneal glucose tolerance test confirms significant glucose intolerance in HFD-fed control animals and a normal glucose sensitivity in animals with Sod3 gene transfer (Figure 5b). A direct measurement of serum insulin levels (Figure 5c) and the results from insulin tolerance test (Figure 5d) suggest that HFD-fed control mice have developed insulin resistance at the end of the 8-week HFD feeding. In contrast, animals that underwent Sod3 gene transfer have normal insulin levels and exhibit the same insulin sensitivity as regular mice fed a regular chow (Figure 5e).

Sod3 gene transfer affects expression of critical genes involved in inflammation and energy metabolism

A decrease in crown-like structures in white adipose tissue (Figure 3e) exposed to sod3 gene transfer in HFD-fed animals
suggests that overexpression of the Sod3 gene may suppress HFD-induced inflammation in adipose tissue. To test this possibility, we compared the mRNA levels of genes with known functions in inflammation, including F4/80, Tnfa, Cd11c, Mcp1 and Il6. Results in Figure 6a show higher mRNA levels of the selected genes in white adipose tissue in obese mice injected with control plasmid. As expected, mRNA levels of the same group of genes in animals exposed to Sod3 gene transfer are identical to that of regular mice. The mRNA levels of the adiponectin gene in obese mice appear lower than that in regular mice and those exposed to Sod3 gene transfer (Figure 6b). The mRNA levels in white adipose tissue of the leptin gene are lower in mice injected with the Sod3 gene, in contrast to a significant increase in Il4 mRNA level. Similar experiments were performed in the mouse liver to assess the effects of Sod3 gene transfer on HFD-fed animals on expression of genes involved in lipid and glucose metabolism. With the

Figure 3. Sod3 gene transfer suppresses fat accumulation and represses crown-like structure in adipose tissue. (a) Images of different fat pads, one kidney was included when photographed the perirenal white adipose tissue; (b) weight of adipose tissues; (c) images of H&E staining of EWAT, IWAT, PWAT and BAT; (d) average diameter of adipocytes in white adipose tissue calculated from 500 randomly selected cells from five tissue slides; (e) number of crown-like structures in white adipose tissue collected from different locations. □ Animals fed a regular Chow; ▪ HFD-fed mice injected with pLIVE-SEAP; and ■ HFD-fed mice injected with pLIVE-SOD3. Data represent mean ± s.e.m. (n = 5). *P < 0.05 compared with chow mice; # P < 0.05 compared with pLIVE-SEAP-injected animals. BAT, brown adipose tissue; EWAT, epididymal white adipose tissue; IWAT, inguinal white adipose tissue; PWAT, perirenal white adipose tissue.
exception of Acc, the mRNA levels of genes involved in de novo lipogenesis in the liver, including Srebp1c, Fas and Scd1, are higher in the two HFD-fed animal groups (Figure 6c). mRNA levels of genes involved in lipolysis and energy expenditure, including Cpt1a, Cpt1β, Pgc1a, Pgc1β and Ucp2, are significantly higher in animals that underwent Sod3 gene transfer than those of HFD-fed control animals (Figure 6d). Expression of genes involved in glucose metabolism, such as G6p, a key gene for gluconeogenesis, is decreased in HFD-fed mice, whereas mRNA levels of Pdk4, a key gene repressing glucose oxidation, are lower in animals that
underwent Sod3 gene transfer compared with that of HFD-fed control mice. The mRNA levels of other genes involving in glucose metabolism, such as Glut4 and Pepck, are not statistically different among the three groups of animals (Figure 6e).

**DISCUSSION**

Chronic inflammation is closely associated with obesity, and an increase in fat intake fuels the reactive oxygen species generation in the adipose tissue and boosts macrophage infiltration. We have previously shown that intraperitoneal injections of clodronate liposomes to eliminate macrophages block HFD-induced weight gain and insulin resistance, although the exact molecular mechanism responsible for the response is not fully understood. In the current work, we investigate the impacts of overexpression of the Sod3 gene focusing the influence of SOD3 on HFD-induced obesity and development of insulin resistance and fatty liver. We used a hydrodynamics-based procedure to obtain a high level of SOD3 gene transfer (Figure 2c), in agreement with the observation that the function of SOD3 is specific in blocking adipogenesis.

It has been previously shown that pro-inflammatory cytokines such as tumor necrosis factor-α (TNFα), monocyte chemoattractant protein 1 (MCP1) and IL6 are associated with the development of obesity, and anti-inflammatory cytokines, such as adiponectin, inhibit the process. At the pro-inflammatory state, macrophages are converted from the M2 (anti-inflammatory state) to M1 type (inflammatory state). M1 macrophages, which express F4/80/Cd11c, will surround the hypertrophic or necrotic adipocytes and form crown-like structures, causing the adipose tissue remodeling and pathological fat pad expansion. In full agreement with these previous studies, our data show that in HFD-fed control mice, there were more macrophages in the adipose tissue (Figures 3c and e) with an increase in transcription of pro-inflammatory marker genes such as Tnfα, Mcp1, Il6, F4/80 and Cd11c (Figure 6a). In contrast, mice transferred with Sod3 gene showed a lower mRNA level of the same set of inflammatory genes and an elevation of adiponectin and anti-inflammatory cytokines, such as adiponectin, inhibit the process.

In this study, we observed that Sod3 gene transfer reduced the inflammation and enhances the energy expenditure. Animals were killed at the end of 8-week feeding (Figure 1b). H&E staining of liver sections and examination of blood concentrations of alanine aminotransferase and aspartate aminotransferase confirm that the hydrodynamics-based procedure for gene transfer is safe (Figures 1c–e).

Sustained high levels of SODs resulted in suppression of HFD-induced weight gain in animals compared with control animals injected with the control plasmid (Figures 3a–d). These results suggest that the function of SOD3 is specific in blocking adipogenesis.

Figure 6. Sod3 gene transfer reduces the inflammation and enhances the energy expenditure. Animals were killed at the end of 8-week feeding and tissue from white adipose tissue and liver were collected for RNA isolation, mRNA levels of selected gene were quantified by real-time PCR. (a) Relative mRNA levels of inflammation-related genes in adipose tissue; (b) relative mRNA levels of anti-inflammatory genes in adipose tissue; (c) relative mRNA levels of genes involved in de novo lipogenesis in the liver; (d) relative mRNA levels of genes involved in lipolysis and energy metabolism in the liver; and (e) relative mRNA levels of genes involved in glucose metabolism in the liver. Animals fed a regular chow; (■) HFD-fed mice injected with pLIVE-SEAP; and (□) HFD-fed mice injected with pLIVE-SOD3. Data represent mean ± s.e.m. (n = 5). *P < 0.05 compared with chow mice; †T < 0.05 compared with pLIVE-SEAP-injected animals.

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Adipose tissue inflammation is a critical determinant leading to the development of insulin resistance and type 2 diabetes. Insulin resistance results in the decrease in glucose oxidation, which leads to glucose intolerance and hyperinsulinemia. Previous studies have shown that the plasma SOD3 level was inversely related to fasting glucose level, body mass index and insulin resistance. A study from Tamai et al. has shown that the genetic variant of the Sod3 gene was associated with insulin resistance and the susceptibility to type 2 diabetes; a missense mutation (Ala40Thr) showed diagnosis of type 2 diabetes at an earlier age and lower insulin sensitivity. Overexpression of SOD3 and other two endogeneous antioxidant enzymes (SOD1 and glutathione peroxidase) protected pancreatic islets from oxidative stress. Factors such as the C/EBP (enhancer-binding protein) enhancer, which regulate the expression of genes involved in insulin resistance, could also elevate gene expression and protein levels of SOD3, which was thought a compensatory effect for improving insulin sensitivity. Our data are in agreement with the conclusions derived from these previous studies and are in support that overexpression of the Sod3 gene could prevent the HFD-induced insulin resistance (Figure 5).

Fatty liver is a common complication of obesity. Previous studies have shown that the SOD3 level is associated with fatty liver and related diseases. Madan et al. showed that SOD3 levels in serum decreased in the patients with fatty liver disease compared with that in the healthy control. Similar results were obtained in obese rats. Inversely, injection of high doses of recombinant SOD3 vectors (3 x 10^10 plaque-forming unit) protected the liver from injury induced by oxidative stress in rats. Some antioxidants such as resveratrol decreased the severity of nonalcoholic fatty liver disease in rats, which was accompanied with elevation of SOD3. High SOD3 levels also provided beneficial effects against nonalcoholic steatohepatitis and liver cirrhosis. In accordance with these data, our results demonstrate that overexpression of the Sod3 gene suppresses the ectopic fat accumulation in the liver and protects the liver from HFD-induced development of fatty liver (Figure 4).

Previous studies have shown that adiponectin gene expression was significantly reduced in ob/ob mice, monkeys and humans. A decrease in serum levels of adiponectin correlates with insulin resistance and glucose intolerance. Adiponectin, in addition to the anti-inflammatory effect, has beneficial effects on the metabolic disorders. It can modulate the expression of Cpt1, Pgc1α and Ucp2, a set of genes involved in energy expenditure. Our results in Figure 6d demonstrate that SOD3 overexpression significantly increased expression of Cpt1α, Cpt1β, Pgc1α, Pgc1β and Ucp2 genes responsible for energy expenditure. No statistical difference was seen in Sod3-injected and HFD-fed control animals with respect to the mRNA levels of genes involved in cholesterol synthesis (Srebp1c), fatty acid biosynthesis (Acc, Fas, Scd1) and glucose metabolism (G6p, Glut4, Pepck). Significant elevation in mRNA levels of the Pdk4 gene was seen in HFD-fed control mice and its level was reduced by Sod3 gene transfer. Further studies are needed to identify genes and metabolic pathways by which the SOD3 applies its effect on the prevention of HFD-induced weight gain, insulin resistance and the development of a fatty liver.

In summary, this study, we demonstrate that overexpression of the Sod3 gene produces a variety of beneficial effects against diet-induced obesity and its complications, including fatty liver and insulin resistance. The protective effect is achieved by suppressing expression of genes responsible for inflammation in adipose tissue and increasing expression of genes for energy expenditure. Our findings also demonstrate that overexpression of SOD3 via gene transfer could result in a protective effect against the development of obesity and obesity-associated insulin resistance and fatty liver and be considered an effective approach in preventing HFD-induced obesity. If same level of SOD3 gene expression can be achieved in humans, the strategy employed in this study could lead to effective prevention of obesity resulted from a HFD.

**MATERIALS AND METHODS**

**Plasmid vectors**

Plasmids carrying either mouse Sod3 or SEAP gene were cloned into a pLIVE plasmid vector from Mirus Bio (Madison, WI, USA). The mouse Sod3 gene was purchased from Thermo Fisher Scientific Biosciences (Middletown, VA, USA), digested with restriction enzymes SacI and XhoI, and the Sod3-containing fragment inserted into multiple cloning sites of the pLIVE vector. The insertion in the new plasmid was confirmed by DNA sequencing (University of Georgia Genomics Facility). The plasmids were isolated using the method of cesium chloride–ethidium bromide gradient centrifugation and kept in saline at −80°C until use.

**Animals and animal experiments**

C57BL/6 mice (male, 9 weeks old, 24–26 g) were purchased from Charles River Laboratories (Wilmington, MA, USA) and housed under standard conditions with a 12 h−12 h light−dark cycle. The HFD (60% calories from fats, 20% from carbohydrates and 20% from proteins) was purchased from Bio-Serv (Frenchtown, NJ, USA; #F3282). Three groups of mice were employed (n = 5 for each), one group was fed normal chow and the remaining two groups were fed a HFD. HFD-fed animals received hydrodynamic injections of either pLIVE-SEAP plasmid (control) or pLIVE-SOD3 plasmids. Mice were fed continuously for 8 weeks and the body weight and composition, and food intake were measured weekly. One week before being killed, animals were fasted for 6 h followed by a glucose tolerance test. The same animals were allowed to rest for 2 days before being fasted for 4 h before an insulin sensitivity test. Animals were killed at the end of the 8th week and blood and various tissue samples were collected for biochemical and histological examinations.

**Assessment of Sod3 gene expression**

Animals were hydrodynamically injected via the tail vein with 10 µg per mouse of pLIVE-SOD3 or pLIVE-SEAP plasmid DNA per mouse on day 1 according to the standard procedure. Approximately 50 µl of blood was collected from the tail using Microvette (Newton, NC, USA) at different times and serum was prepared. SOD3 activity in the blood was determined using a previously established procedure by McCord et al. Reagents for the SOD3 enzyme assay, including xanthine, xanthine oxidase, cytochrome C1 and SOD standard, were purchased from Sigma (St Louis, MO, USA).

**Real-time PCR for mRNA analysis**

Animals were killed at desirable times and the heart, lung, liver, spleen, kidneys and adipose tissues were collected. TRIzol reagents from Invitrogen (Carlsbad, CA, USA) were used for extraction of total RNA from all tissues with the exception of the adipose tissue where the specialized kits from Qiagen (Valencia, CA, USA) for RNA isolation were used. Complementary DNA was synthesized using a First Strand cDNA Synthesis System for quantitative real-time PCR, the commercial kits from OriGene (Rockville, MD, USA). Real-time PCR was performed with SYBR Green as detection reagent and the ΔΔCt method for data analysis. Glyceraldehyde-3-phosphate dehydrogenase mRNA was used as an internal control. All primers (Table 1) were ordered from Sigma. Melting curve analysis of all quantitative PCR products was performed and showed a single DNA duplex.

**Determination of plasma concentrations of alanine aminotransferase, aspartate aminotransferase and insulin**

Blood samples were collected from the heart cavities immediately after the killing mice. Sera were isolated by centrifugation at 5000 r.p.m. for 5 min. alanine aminotransferase and aspartate aminotransferase concentrations were determined following the manufacturer’s instruction (Thermo Fisher Scientific). Insulin levels in the blood were measured using a commercial ELISA kit from Meredia Inc. (Winston Salem, NC, USA).
Measurement of triglycerides, total cholesterol and nonesterified fatty acid in the liver

The measurement was performed according to the Folch method. In brief, ~100 mg of liver sample was freshly prepared and homogenized in a tube containing 20× of liver volume of solution consisting of chloroform and methanol (2:1). Tissue homogenates were incubated at 4 °C overnight and centrifuged at 12,000 r.p.m. for 20 min. Supernatants were collected, air dried and dissolved in 1% Triton-X. Amounts of triglycerides, total cholesterol and nonesterified fatty acid were determined using commercial kits from Thermo Fisher Scientific, Genzyme (Boston, MA, USA) and Wako (Tokyo, Japan), respectively.

Intraperitoneal glucose tolerance test and insulin tolerance test

Mice were fasted for 6 h before intraperitoneal glucose tolerance test. Glucose in 0.9% saline was injected (i.p.) at 2 g kg⁻¹, and the time point was set as 0 min. Blood glucose levels at 0, 30, 60 and 120 min were measured using glucose test strips and glucose meters. For insulin tolerance test, mice were fasted for 4 h before injection of insulin (humulin, 0.8 U kg⁻¹) from Eli Lilly (Indianapolis, IN, USA), and blood glucose was measured at 0, 30, 60 and 120 min after insulin injection. A HOMA-IR (homeostasis model for assessing insulin resistance) was employed to define the degree of insulin resistance. [HOMA-IR = fasting glucose (mg dl⁻¹) x fasting insulin (μU ml⁻¹) / 22.5].

H&E staining

Freshly collected tissues from animals were fixed overnight in 10% neutral formalin solution. After dehydration and embedding, tissue sections were made 5–7 μm in thickness and dried at 37 °C for 1 h. H&E staining was performed according to the instruction of a commercial kit from BBC media thickness. An optical microscope (ECLIPSE Ti; Nikon) was used to examine the slides and selected structures were photographed.

Statistical analysis

Statistical analysis was performed using one-way analysis of variance, and a P-value below 0.05 (P < 0.05) is considered statistically different. Data were expressed as mean ± s.e.m.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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