Original Article

Type 2 diabetes model TSOD mouse is exposed to oxidative stress at young age

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Tsumura Suzuki Obese Diabetes (TSOD) mouse, a model of obese type 2 diabetes, older than around 11 weeks of age develops diabetic phenotypes. Previous studies have indicated that the development of diabetes is partly due to three loci associated with body weight and glucose homeostasis. However, little is known about the initial events triggering the development of the diabetic phenotypes in TSOD mouse. Here, we investigated the alteration of diabetes-related parameters, including the levels of blood glucose and inflammatory cytokines, and the oxidative stress status, in young TSOD mice. TSOD mice at 5 weeks of age showed increases in body weight and plasma total cholesterol level, but not hyperglycemia or impaired glucose tolerance, compared with age-matched control Tsumura Suzuki Non-Obese (TSNO) mice. Plasma tumor necrosis factor (TNF)-α and interleukin (IL)-6 were not detected in TSOD mice at 5 weeks of age. However, plasma total hydroxyoctadecadienoic acid (tHODE), a biomarker of oxidative stress, was increased in TSOD mice relative to TSNO mice at same age. The results demonstrated that young TSOD mice are exposed to oxidative stress before developing the diabetic phenotypes, and suggested that oxidative stress is an initial event triggering the development of diabetes in TSOD mice.

Key Words: type 2 diabetes, oxidative stress, hyperglycemia, inflammation, TSOD mouse

Type 2 diabetes mellitus is a common chronic disease that has seen a continuous increase in prevalence, because of reduced physical activity and increased obesity caused by lifestyle changes.[1] It is estimated that the global prevalence of diabetes would increase from 171 million in 2000 to 366 million in 2030.[2] The pathogenesis of type 2 diabetes is characterized by the progressive decline in insulin action (insulin resistance) in peripheral tissues, accompanied by the impairment of insulin secretion in pancreatic β cells to compensate for the insulin resistance, leading to overt hyperglycemia.[3] Numerous studies have suggested that the development of insulin resistance and pancreatic β cell dysfunction is induced by complicated and coordinated events between oxidative stress and inflammation.[4] As the development of type 2 diabetes is promoted by complex interactions between genetic factors, i.e., multiple susceptibility genes, and environmental factors,[3] it is difficult to analyze the biochemical and molecular mechanisms underlying the development of diabetes in human.[1] On the other hand, because it is easier to control the experimental conditions for rodent than human, rodent is widely used to investigate human diseases. In this regard, animal models of type 2 diabetes, which represent the complex nature of human type 2 diabetes, are useful in biomedical studies and expected to contribute to new insights into human diabetes.

Tsumura Suzuki Obese Diabetes (TSOD) mouse has been established as a polygenic model of obese type 2 diabetes by selective breeding of ddY mice.[5] TSOD mice developed obesity, hyperphagia, hyperinsulinemia, and hyperleptinemia before developing the diabetic phenotypes, compared with age-matched control Tsumura Suzuki Non-Obese (TSNO) mice.[5–7] Several diabetes-related phenotypes, including hypertriglyceridemia, hypercholesterolemia, impaired glucose tolerance, insulin resistance, impaired insulin secretion, and hyperglycemia, were apparently observed in TSOD mice older than around 11 weeks of age.[5–8] It is suggested that the insulin resistance in TSOD mice is partly due to the impairment of insulin-stimulated translocation of glucose transporter type 4 (GLUT4).[8]

As most cases of human type 2 diabetes are attributed to the interactions between environmental factors and genetic factors, namely, multiple susceptibility genes,[3] it is believed that type 2 diabetic models with polygenic mutations may provide more similar phenotypes to human type 2 diabetes than the diabetic models with monogenic mutation.[9] As has been reported previously, distinct traits related to body weight and insulin and glucose levels in TSOD mouse are affected by three gene loci: non-insulin-dependent diabetes (*Nidd*) 4 on mouse chromosome 11, which is associated with glucose level, *Nidd5* on mouse chromosome 2, which is associated with insulin level and body weight, and *Nidd6* on mouse chromosome 1, which is associated with body weight.[6] Although it is considered that the three gene loci partly contribute to the development of diabetes in TSOD mouse, little is known about the initial events triggering the development of the diabetic phenotypes in TSOD mouse.

In the present study, we investigated the alteration of diabetes-related parameters, including blood glucose, inflammatory cytokines, and lipid oxidation products, which are used as biomarkers of oxidative stress *in vivo*, in TSOD mice at 5 weeks of age. We found that body weight and plasma total cholesterol level, but not glucose or inflammatory cytokine levels, were increased in TSOD mice at 5 weeks of age compared with age-matched TSNO mice. Moreover, we demonstrated that lipid oxidation products were significantly increased in TSOD mice at 5 weeks of age compared with age-matched TSNO mice, indicating that TSOD mice were already exposed to oxidative stress at a young age. The results suggest that the increased oxidative stress is an initial event that triggers the development of diabetes in TSOD mouse.

Materials and Methods

Materials.   13-Hydroxy-9*Z*,11*E*-octadecadienoic acid [13-(*Z,E*)-HODE], 9-hydroxy-10*E*,12*Z*-octadecadienoic acid [9-(*Z,E*)-HODE], and 13S-hydroxy-10*E*,12*Z*-octadecadienoic-9,10,12,13-d4 acid (13-HODE-d4) were obtained from Cayman Chemical Com-

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pany (Ann Ardor, MI). 9-Hydroxy-10*E*,12*E*-octadecadienoic acid [9-(*E*,*E*)-HODE] and 13-hydroxy-9*E*,11*E*-octadecadienoic acid [13-(*E*,*E*)-HODE] were obtained from Larodan Fine Chemicals AB (Malmo, Sweden). Other materials were of the highest grade commercially available.

Animals. Male TSOD and control male TSNO mice were obtained from the Institute for Animal Reproduction (Ibaraki, Japan). The animals were housed individually and had free access to food (CE-2; Clea Japan Inc., Tokyo, Japan) and water. The animal room was maintained at $23 \pm 2°C$ and $50 \pm 10\%$ humidity under a 12 h light (8:00–20:00) and dark (20:00–8:00) cycle. The animals were acclimated to the laboratory environment for at least one week before the experiment. The experimental protocols were approved by the Institutional Animal Care and Use Committee of the National Institute of Advanced Industrial Science and Technology.

Measurement of blood glucose and plasma cytokines. Non-fasting blood glucose level was measured around 17:00. Mice were retained in a mouse restrainer and their tail tips were resected with surgical scissors. Glucose level in effused blood (approximately 1 µl) was measured with a glucose meter (Life Check; EIDIA Co. Ltd., Tokyo, Japan). For the measurement of plasma cytokine levels, mice were sacrificed after fasting for 16 h and blood was collected from the vena cava. The collected blood was gently mixed with heparin and centrifuged. The obtained plasma samples were stored at $-30°C$ until use. Plasma tumor necrosis factor (TNF)-α and interleukin (IL)-6 levels were measured with an ELISA kit (eBioscience, Inc., San Diego, CA) according to the manufacturer's instructions.

Oral glucose tolerance test (OGTT). After fasting for 16 h, glucose solution at the dose of 1.5 g/kg body weight was administered orally with a gavage needle. Blood glucose levels in tail vein were measured by the above-mentioned method at 0 (pre-administration), 15, 30, 60, and 120 min after glucose administration using a glucose meter.

Analysis of plasma total hydroperoxyoctadecadienoic acid level by liquid chromatography-tandem mass spectrometry (LC-MS/MS). HODEs were measured with a previously described method.[10,11] Briefly, mouse plasma sample was mixed with the internal standard, 13-HODE-d_4, and then butylated hydroxytoluene (BHT) and triphenylphosphine (TPP) were added to reduce the hydroperoxides in the sample. The reduced sample was saponified by adding KOH in methanol. The mixture was acidified with 10% acetic acid in water and extracted with chloroform/ethyl acetate (4:1, v/v). The chloroform/ethyl acetate layer was concentrated and divided equally into two portions, and each portion was evaporated to dryness under nitrogen. One portion of the derivatized sample was reconstituted with methanol/water (70:30, v/v), and subjected to LC-MS/MS analysis of HODEs. Another portion of the derivatized sample was used for the measurement of plasma cholesterol and cholesteryl esters, which are assessed as total cholesterol, by gas chromatography-mass spectrometry (GC-MS). The precursors, the product ions, and the collision energies were determined after the optimization of MS/MS conditions as follows: m/z = 295.0 and 194.6–195.6 at 21 eV for both 13-(*Z*,*E*)-HODE and 13-(*E*,*E*)-HODE; m/z = 295.0 and 170.5–171.5 at 24 eV for both 9-(*Z*,*E*)-HODE and 9-(*E*,*E*)-HODE; and m/z = 299.0 and 197.6–198.6 at 15 eV for 13-HODE-d_4. Plasma total hydroxyoctadecadienoic acid (tHODE) levels were determined from the total amount of the products of the free-radical-mediated oxidation of the four isomers: 13-(*Z*,*E*)-HODE, 13-(*E*,*E*)-HODE, 9-(*Z*,*E*)-HODE, and 9-(*E*,*E*)-HODE. Cholesterol was identified on the basis of the retention times and the mass spectra; ions having m/z = 458 were selected for the quantification. Outliers were omitted by the Smirnov-Grubbs' outlier test.

Statistics. The results were expressed as means $\pm$ SE. Statistical analysis was performed by using analysis of variance (ANOVA) followed by Tukey's test for multiple comparisons with Ekuseru-Toukei 2012 (Social Survey Research Information Co., Ltd.). The comparison of two groups was carried out by the unpaired *t* test. Differences with a probability of 5% or less were considered significant.

Results

Body weight and biochemical parameters of TSNO and TSOD mice. We examined body weight and several biochemical parameters related to diabetes in TSNO and TSOD mice at 5 and 11 weeks of age. The body weights of TSOD mice at 5 and 11 weeks of age were significantly higher than those of age-matched TSNO mice (Fig. 1a), consistent with a previous report.[7] Weight gain in TSOD mice during the period between 5 and 11 weeks of age was much greater than that in TSNO mice during the same period, indicating that the progression of obesity is rapid in TSOD mice compared with TSNO mice. Whereas plasma total cholesterol levels in TSOD mice at 5 and 11 weeks of age were significantly higher than those in age-matched TSNO mice, no significant difference was observed between TSOD mice at 5 and 11 weeks of age (Fig. 1b), indicating that TSOD mice already developed hypercholesterolemia at 5 weeks of age. Although non-fasting blood glucose level in TSOD mice at 11 weeks of age was significantly higher than that in age-matched TSNO mice, as reported previously,[7] no significant difference in glucose level was found between TSNO and TSOD mice at 5 weeks of age (Fig. 1c). Taken together, the results indicate that TSOD mice at 5 weeks of age develop obesity and hypercholesterolemia, both of which contribute to the development and/or progression of type 2 diabetes, without significant increases in blood glucose level.

Oral glucose tolerance test in TSNO and TSOD mice. OGTT is widely used for the determination of diabetes in human. To examine whether young TSOD mice developed impaired glucose tolerance, we performed OGTT in TSNO and TSOD mice at 5 and 11 weeks of age. At 5 weeks of age, blood glucose levels before and after glucose loading were not significantly different between TSNO and TSOD mice, except for the glucose level at 120 min after glucose loading (Fig. 2a). In contrast, at 11 weeks of age, blood glucose levels before and after glucose loading were significantly increased in TSOD mice compared with TSNO mice (Fig. 2b), consistent with a previous study,[12] indicating that glucose tolerance in TSOD mice is normal at 5 weeks of age, but is gradually exacerbated thereafter.

Plasma TNF-α and IL-6 in TSNO and TSOD mice. Inflammatory cytokines TNF-α and IL-6 play a key role in the development of insulin resistance, which leads to impaired glucose tolerance in prediabetes and subsequent overt type 2 diabetes.[13] We therefore measured TNF-α and IL-6 levels in TSNO and TSOD mice at 5 and 11 weeks of age. As shown in Fig. 3, whereas TNF-α and IL-6 were not detected in TSOD mice at 5 weeks of age, both inflammatory cytokines showed marked increases in TSOD mice at 11 weeks of age, indicating that TSOD mice developed inflammation at 11 weeks of age, but not at 5 weeks of age.

Lipid oxidation products in TSNO and TSOD mice. Oxidative stress, which is induced by the excessive production of reactive oxygen species (ROS) and/or the decrease in antioxidants, promotes the development of insulin resistance and the impairment of insulin secretion in obese subjects.[14] Exacerbation of oxidative-stress-derived inflammation is suggested to be the major mechanism underlying the pathogenesis and progression of obesity-related diseases.[15] To determine whether young TSOD mice were exposed to oxidative stress, we measured plasma levels of tHODE, which is composed of lipid oxidation products derived from linoleate, and is used as a biomarker of oxidative stress status *in vivo*.[10,11] At 11 weeks of age, plasma tHODE level in TSOD mice was significantly higher than that in TSNO mice (Fig. 4). Interestingly, plasma tHODE level in TSOD mice at 5 weeks of age was already higher and comparable to that in TSOD
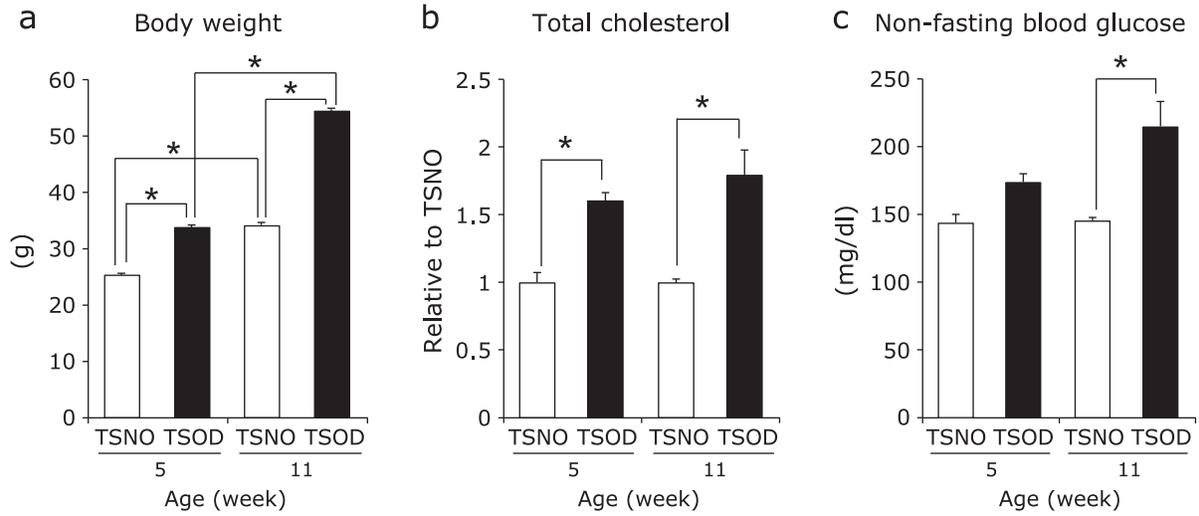
Fig. 1. Changes in body weight (a), plasma total cholesterol (b), and non-fasting blood glucose (c) in TSNO (white bar) and TSOD (black bar) mice. Plasma total cholesterol level in TSNO mice set to 1. Results are expressed as means ± SE (n = 3–6). Statistical analyses were carried out using ANOVA (Tukey's post-hoc test). *$p<0.05$.
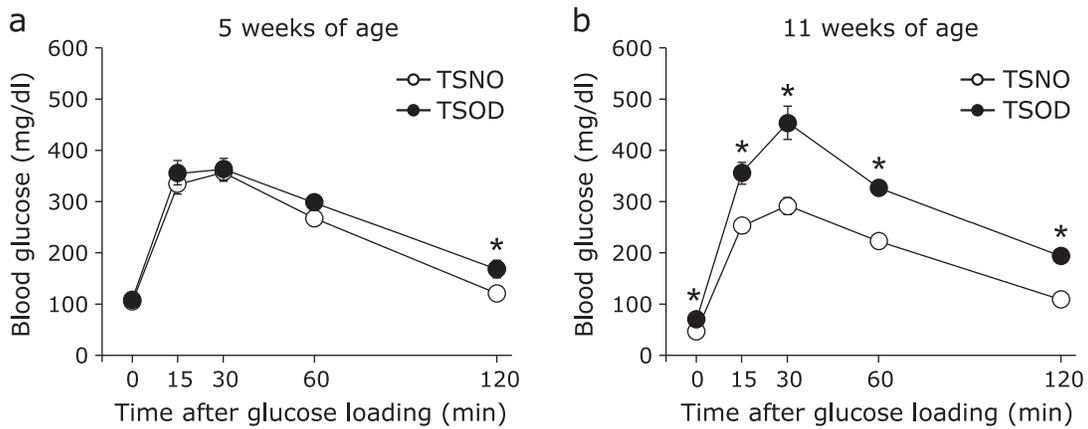
Fig. 2. OGTT in TSNO and TSOD mice. Glucose levels before (0 min) and after (15, 30, 60, and 120 min) oral glucose loading in TSNO (white circle) and TSOD (black circle) mice at 5 (a) and 11 (b) weeks of age. Results are expressed as means ± SE (5 weeks of age: n = 11–12; 11 weeks of age: n = 6 each). Statistical analyses were carried out using the unpaired t test. *Significant difference from TSNO mice ($p<0.05$).
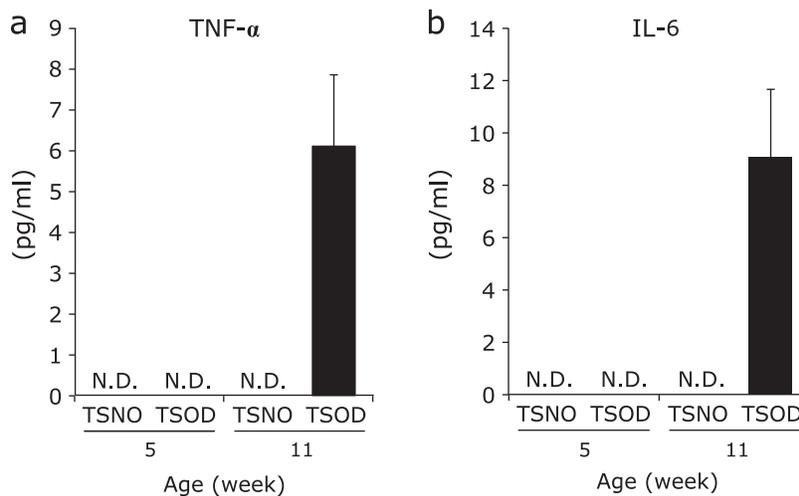
Fig. 3. Changes in fasting plasma TNF-α (a) and IL-6 (b) levels in TSNO (white bar) and TSOD (black bar) mice. Results are expressed as means ± SE (n = 6 each). N.D. indicates not detected.
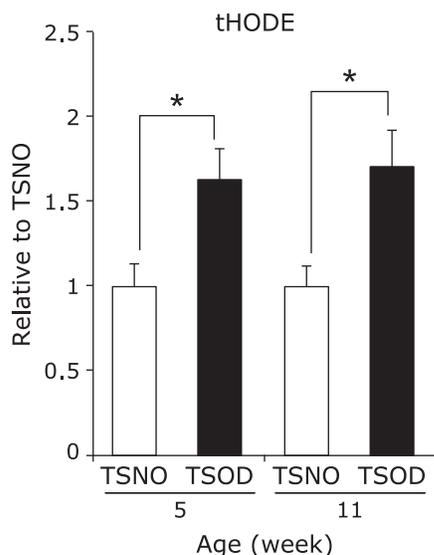
Fig. 4. Plasma lipid oxidation products in TSNO (white bar) and TSOD (black bar) mice. Plasma tHODE level in TSNO mice was set to 1. Results are expressed as means ± SE (5 weeks TSNO and TSOD: $n = 5$ each; 11 weeks TSNO: $n = 6$; 11 weeks TSOD: $n = 3$). Statistical analyses were carried out using the unpaired t test. *Significant difference from age-matched TSNO mice ($p < 0.05$).

mice at 11 weeks of age. The results demonstrate that TSOD mice are already exposed to oxidative stress at 5 weeks of age before developing the type 2 diabetic phenotypes.

Discussion

In the present study, we investigated diabetes-related parameters in TSOD mice at 5 and 11 weeks of age. We showed that TSOD mice at 11 weeks of age developed obesity, hypercholesterolemia, impaired glucose tolerance, and hyperglycemia compared with TSNO mice (Fig. 1 and 2), consistent with previous studies.[5,7,12] Those findings suggest that TSOD mice at 11 weeks of age have type 2 diabetic symptoms. On the other hand, TSOD mice at 5 weeks of age developed obesity and hypercholesterolemia, but not hyperglycemia or impaired glucose tolerance. Together, our results suggest that TSOD mice at 5 weeks of age are prediabetic and at a high risk of type 2 diabetes.

As shown in Fig. 1b, plasma total cholesterol level in TSOD mice at 5 weeks of age was higher than that in age-matched TSNO mice, whereas Miyata *et al.*[7] reported that cholesterol level in TSOD mice at 1 month of age was equivalent to that in age-matched TSNO mice. It is known that cholesterol level is greatly affected by food consumption.[16] Whereas a previous study demonstrated that food consumption in TSNO and TSOD mice at 1 month of age was around 3.0 g/day,[7] in this study, food consumption in TSOD mice at 5 weeks of age was 6.7 ± 0.1 g/day, which was significantly higher than that in age-matched TSNO mice (data not shown). Thus, the discrepancy of the cholesterol level is due to the difference in the amount of food consumed by young TSOD mice.

In the progression of type 2 diabetes, oxidative stress has been proposed as the major factor underlying the development of insulin resistance and the impairment of pancreatic insulin secretion, and the subsequent elevation of blood glucose level.[14,15] To examine whether TSOD mice were exposed to oxidative stress, we measured plasma tHODE levels at 5 and 11 weeks of age. We found that TSOD mice at 5 weeks of age were already exposed to oxidative stress before developing the type 2 diabetic phenotypes (Fig. 4). At this age, we and others observed that the TSOD mice also developed obesity (Fig. 1a).[7] It has been reported that free fatty acids released from adipose tissue accelerate mitochondrial uncoupling and β oxidation, leading to the production of ROS.[13,15] ROS, in turn, are thought to induce adipocyte hypertrophy, which contributes to the progression of obesity.[17] Numerous reports have suggested that oxidative stress and obesity are exacerbated when those two factors interact with each other.[18,19] Although further studies are required to elucidate the mechanism underlying the induction of oxidative stress in TSOD mice, our findings suggest that oxidative stress in young TSOD mice is attributed to ROS production associated with obesity.

It is known that ROS induce the production of inflammatory cytokines, including TNF-α and IL-6, through the activation of nuclear factor-κB, a major transcription factor involved in inflammation.[20] In this study, we demonstrated that plasma TNF-α and IL-6, which play an important role in the development of type 2 diabetes, were not detected in TSOD mice at 5 weeks of age (Fig. 3), consistent with a previous report,[21] even though TSOD mice were already exposed to oxidative stress. We also showed that blood glucose level and impaired glucose tolerance in TSOD mice at 5 weeks of age were not significantly different from those in age-matched TSNO mice (Fig. 1c and 2). The results imply that the increased oxidative stress in TSOD mice at 5 weeks of age is not sufficient to induce hyperglycemia and inflammation. On the other hand, in TSOD mice at 5 and 11 weeks of age, whereas plasma tHODE levels were almost the same (Fig. 4), TNF-α and IL-6 levels were markedly different (Fig. 3), and diabetic phenotypes, including obesity, blood glucose level (Fig. 1), and impaired glucose tolerance (Fig. 2), were exacerbated during the period between 5 and 11 weeks of age. The results underscore the possibility that the progression of diabetes in TSOD mice is caused by the long-term exposure to oxidative stress.

In conclusion, we demonstrated that oxidative stress precedes marked inflammation, impaired glucose tolerance, and hyperglycemia in TSOD mice, suggesting that oxidative stress triggers the development of diabetic phenotypes with systemic inflammation. TSOD mouse may be suitable animal model for the analysis of oxidative-stress-mediated pathological alterations in type 2 diabetes.

Conflict of Interest

No potential conflicts of interest were disclosed.

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