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Dietary trimethylamine *N*-oxide exacerbates impaired glucose tolerance in mice fed a high fat diet

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Trimethylamine *N*-oxide (TMAO) is an oxidation product of trimethylamine (TMA) and is present in many aquatic foods. Here, we investigated the effects of TMAO on glucose tolerance in high fat diet (HFD)-fed mice. Male C57BL/6 mice were randomly assigned to the control, high fat (HF), and TMAO groups. The HF group was fed a diet containing 25% fat, and the TMAO group was fed the HFD plus 0.2% TMAO for 4 weeks. After 3 weeks of feeding, oral glucose tolerance tests were performed. Dietary TMAO increased fasting insulin levels and homeostasis model assessment-estimated insulin resistance (HOMA-IR) and exacerbated the impaired glucose tolerance in HFD-fed mice. These effects were associated with the expression of genes related to the insulin signal pathway, glycogen synthesis, gluconeogenesis and glucose transport in liver. mRNA levels of the pro-inflammatory cytokine MCP-1 increased significantly and of the anti-inflammatory cytokine IL-10 greatly decreased in adipose tissue. Our results suggest that dietary TMAO exacerbates impaired glucose tolerance, obstructs the hepatic insulin signaling pathway, and causes adipose tissue inflammation in mice fed a high fat diet.

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[**Key words:** Glucose tolerance; Insulin resistance; Inflammation; High fat diet; Trimethylamine *N*-oxide]

Trimethylamine *N*-oxide (TMAO) is an oxidation product of trimethylamine (TMA). Several metabolomic studies have shown depletion of serum or urinary TMAO in HFD-induced obese mice or Zucker rats (1–3), whereas, elevation in TMAO levels has been observed in growth hormone receptor (GHR) mutant obese mice (4). In addition, increased serum and urinary TMAO levels is shown to be associated with the predisposition to impaired glucose homeostasis and NAFLD in HFD-fed mice (5). Previous studies have shown that dietary choline or TMAO can promote atherosclerosis development in apoE deficient mice (C57BL/6J *Apoe*$^{-/-}$) (6). These studies have demonstrated that TMAO is detrimental to health, but the mechanism underlying this effect is not clear. TMAO is a compound abundantly present in marine fish and invertebrates. The TMAO content is 30–90 mmol/kg in the muscle of crustaceans and up to 200 mmol/kg in squids (7,8). The effects of dietary TMAO on lipid metabolism and glycometabolism have not been reported. A recent research indicated that the increased serum TMAO is associated with impaired glucose tolerance induced by a high fat/high cholesterol diet in *Macaca mulatta* (9). However, the influence of dietary intervention with TMAO on IR has been less studied.

A high fat diet (HFD) can induce obesity, which is a condition resulting from an excess of adipose tissue. Obesity, a severe health problem, causes insulin resistance (IR), type 2 diabetes, fatty liver disease, and coronary artery disease (10,11). IR is characterized by impaired glucose tolerance and lipid metabolism (11). Obesity is also known as an inflammatory state (12). Tumor necrosis factor alpha (TNF-α), interleukin-6 (IL-6) and monocyte chemoattractant protein-1 (MCP-1) mediate obesity-induced chronic low-grade inflammation. These cytokines activate inflammatory pathways in adipose tissue, which impair insulin signaling by targeting the downstream components of the insulin signaling pathway (13–16).

In this study, we aimed to investigate the effect of dietary TMAO on HFD-induced impaired glucose tolerance and studied the possible mechanism underlying these effects, for example, the insulin signaling pathway and adipose inflammation.

MATERIALS AND METHODS

Chemicals TMAO was purchased from Sigma (St. Louis, MO, USA).

Animals and diets Male C57BL/6 mice aged 6 weeks were provided by Vital River Laboratories (Beijing, China). The mice were maintained in pathogen-free conditions at constant humidity of 65 ± 15% and temperature of 23 ± 2°C with a 12 h light/dark cycle. After a 1-week adaptation period, the mice were randomly assigned to three groups (8 mice each): control (Con) group, high fat (HF) group and TMAO group. Experimental diets were modified basing on AIN76, as shown in Table S1. All mice had free access to water and food.

After 4 weeks of feeding, mice were sacrificed after 10-h over fasting and blood was collected by orbital venipuncture. Serum was separated from blood sample by centrifuging the blood at 5000 ×*g* for 15 min. Liver, brains, kidneys, hearts, perirenal adipose tissues and epididymal adipose tissues were quickly excised, and washed with ice-cold isotonic saline. After excess water on the surface was removed by blotting with filter paper, the tissues were weighted, frozen in liquid nitrogen and stored at −80°C until analysis. The experiment was reviewed and approved by the ethical committee of experimental animal care at Ocean University of China (approval no. 2009-0007).

Serum and hepatic lipids determination Liver lipids were extracted with chloroform–methanol 2:1 (v/v) as described by Folch et al. (17). The concentrations of serum and hepatic triglyceride (TG) and total cholesterol (TC) were determined using enzymatic reagent kits (Biosino, Beijing, China). Atherosclerosis indices (AIs)

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was calculated by AIs = (TC-HDL-c)/HDL-c (18). Total hepatic phospholipids levels were analyzed by the methods of Bartlett (19).

Serum glucose, insulin and hepatic glycogen determination Serum glucose levels were determined by enzymatic reagent kits (Biosino, Beijing, China) according to the manufacturer's instruction. Serum insulin levels were determined using enzyme-linked immunosorbent assay (ELISA) kits (Millipore, Boston, MA, USA). Homeostasis model assessment of Insulin Resistance (HOMI-IR) was calculated by HOMA-IR = (FI*G)/22.5, where FI is fasting insulin (uU/mL) and G is fasting glucose (mmol/L) (20,21). For glycogen determination, liver tissues (80–100 mg) were homogenized with alkali lye (1:3 w/v [mg:μL]) and heated in boiling water for 30 min. After being cooled in an ice bath, samples were vigorously mixed with 95% alcohol (1:8 w/v [mg:μL]), chilled again and centrifuged at 5500 ×*g* for 30 min. The precipitate was dissolved in 1.5 mL distilled water, and glycogen concentrations were measured using an enzymatic reagent kits (JianCheng, Nanjing, China).

Quantification of TMAO Liquid chromatography with tandem mass spectrometry (LC/MS/MS) was used for quantification of TMAO according to the methods of Wang et al. (6). Plasma proteins were precipitated with four volumes of ice-cold methanol and small-molecule analytes within supernatants were analyzed after injection onto a phenyl column (4.6 × 250 mm, 5 mm SB-Phenyl; Agilent Technologies) at a flow rate of 0.8 ml/min using a Cohesive HPLC interfaced with a PE Sciex API 365 triple quadrupole mass spectrometer (Agilent Technologies). LC gradient (LC1) starting from 10 mM ammoniumformate over 0.5 min, then to 5 mM ammoniumformate, 25% methanol and 0.1% formic acid over 3 min, held for 8 min. TMAO was monitored in positive multiple reaction monitoring (MRM) mode using characteristic precursor–product ion transitions: m/z 76 → 58. Concentrations of TMAO standards were made to prepare the calibration curves for quantification of plasma TMAO.

Oral glucose tolerance test After 3 weeks of feeding, mice were fasted overnight and a basal blood sample (0 min) was collected from the tail vein. The mice were then administered oral glucose (2 g/kg body weight) by gavage, and additional blood samples were collected at 30 and 120 min. Serum glucose levels were determined using the OneTouch Ultra Vue (Johnson, New Buren Zwick, NJ, USA), and the area under the curve for blood glucose (AUC_{GLU}) over 2 h was calculated. AUC was calculated by AUC = 0.25 × A + B + 0.75 × C (A, B, and C represent the blood glucose level at 0, 0.5, and 2 h).

Quantitative RT-PCR analysis of the expression of insulin signaling-related and inflammation-related genes The expression of insulin signal transduction-related genes, such as those for insulin receptor substrate 1(IRS1), insulin receptor substrate 2 (IRS2), phosphatidylinositol 3-kinase (PI3Kr1), protein kinase B (Akt2), forkhead box-containing protein O subfamily-1 (FOXO1), liver-specific glycogen synthase (GYS2), glucose-6-phosphatase (G6pase), phosphoenolpyruvate carboxykinase (PEPCK), glucose transporter-2 (GLUT2), glucose transporter-4 (GLUT4) and inflammation-related genes, such as those for MCP-1, TNF-α, IL-6, IL-10, were examined by reverse transcriptase-polymerase chain reaction (RT-PCR). Total RNA was extracted from 100 mg tissue using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). RNA was quantified by measuring absorbance at 260 nm and its integrity was verified by agarose gel electrophoresis using ethidium bromide for visualization. RNA was converted into cDNA with M-MLV Reverse Transcriptase (Promega, Madison, WI, USA). The concentration of cDNA was analyzed by real-time PCR (Bio-Rad iQ5 Multicolor Real-Time PCR Detection System, Bio-Rad, Hercules, CA, USA) by using Sybr Green I Master Mix (Roche, Basel, Switzerland). The cycling conditions were 95°C for 10 min, annealing for 15 s, 60°C for 20 s and extension at 72°C for 30 s. Primer sequences for amplification are listed in Table S2. Gene expression was determined by relative quantification using the standard curve method. A final melting curve certified the authenticity of the target product. Transcript levels are expressed as the ratio of signal intensity for the target gene to that of 18S.

Inflammation cytokine concentration in the serum Serum levels of inflammation-related cytokines, such as MCP-1, TNF-α, and IL-6 were measured using ELISA kits (R&D Systems, Minneapolis, MN, USA).

Statistics All data were subjected to analysis of variance by using the SPSS software (version 18.0; SPSS Inc., Chicago, IL, USA). Differences between the means were tested using a one-way ANOVA followed by Tukey's test. All values in tables and figures are expressed as mean ± standard error of the mean (SEM). $P < 0.05$ was considered statistically significant.

RESULTS

General observations After 4 weeks of feeding, body weight gain and the weight of the perirenal and epididymal adipose tissue in the HF group were all higher than those in the Con group ($P < 0.05$ for all). However, there were no significant differences in body weight gain, liver weight, perirenal adipose tissue weight, or epididymal adipose tissue weight between the TMAO group and HF group (Table 1).

TABLE 1. Growth parameters, Plasma and Liver chemistries in C57BL/6 mice fed experimental diets.

Group	Con	HF	TMAO
Growth parameters			
Food intake (g/d)	3.10 ± 0.09	3.00 ± 0.10	2.99 ± 0.10
Body weight gain (g)	4.85 ± 0.32	7.62 ± 0.31*	8.12 ± 0.27
Liver weight (mg/g BW)	34.6 ± 2.19	37.4 ± 1.36	37.5 ± 0.86
Perirenal WAT (mg/g BW)	4.83 ± 0.71	10.9 ± 1.35*	10.9 ± 1.75
Epididymal WAT (mg/g BW)	12.2 ± 1.36	22.9 ± 2.70*	25.1 ± 4.07
Visceral WAT (mg/g BW)	17.1 ±2.07	33.8 ± 3.97*	36.0 ± 5.78
Plasma chemistries			
TG (mmol/L)	0.999 ± 0.121	1.62 ± 0.04**	1.43 ± 0.07#
TC (mmol/L)	3.79 ± 0.13	5.31 ± 0.29**	4.13 ± 0.19##
HDL-c (mmol/L)	2.61 ± 0.17	3.29 ± 0.14**	3.23 ± 0.12
AIs	0.452 ± 0.050	0.624 ± 0.096*	0.278 ± 0.024##
Fasting glucose (mmol/L)	7.25 ± 0.61	9.48 ± 0.79*	8.37 ± 0.60
Fasting insulin (μU/ml)	4.22 ± 0.19	5.50 ± 0.25*	9.5 ± 1.8#
HOMA-IR	1.36 ± 0.21	2.32 ± 0.05*	3.53 ± 0.13#
Liver chemistries			
TG (mg/g)	20.52 ± 1.78	30.56 ± 2.57**	31.99 ± 2.08
TC (mg/g)	2.46 ± 0.13	2.61 ± 0.16*	2.91 ± 0.14
PL (mg/g)	30.12 ± 1.96	33.91 ± 1.23*	33.25 ± 0.47
Glycogen content (mg/g)	19.0 ± 1.12	5.76 ± 0.78**	3.5 ± 0.44#

WAT: Weight of adipose tissue. Data are presented as mean ± SEM; $n = 8$ mice per group. *$P < 0.05$, **$P < 0.01$ compared to the control group; #$P < 0.05$, ##$P < 0.01$ compared to the HF group.

Serum TMAO levels There were no significant differences of Serum TMAO levels in Con and HF groups. However, Dietary TMAO significantly increased serum TMAO in TMAO group, compared with HF group (Fig. 1).

Effect of TMAO on serum and hepatic lipid levels Compared with the Con group, serum lipids, the HF group had significantly higher serum lipid levels, hepatic lipid levels, and atherosclerosis indices (AIs).

The TMAO group tended to have lower serum TG and TC concentrations ($P < 0.05$, $P < 0.01$) and AIs ($P < 0.01$) than the HF group. However, there were no significant differences in hepatic lipids between the 2 groups (Table 1).

Effect of TMAO on oral glucose tolerance To investigate the effects of TMAO on glucose tolerance, oral glucose tolerance tests (OGTTs) were carried out at the end of the 3rd week. After 0.5 h of orally administered with glucose (2 g/kg body weight), serum glucose levels were significantly higher in the HF group than in the Con group ($P < 0.01$), and total areas under the glucose curve (AUC_{GLU}) was significantly higher in the HF group ($P < 0.01$). Dietary TMAO significantly increased serum glucose levels at 0.5 h

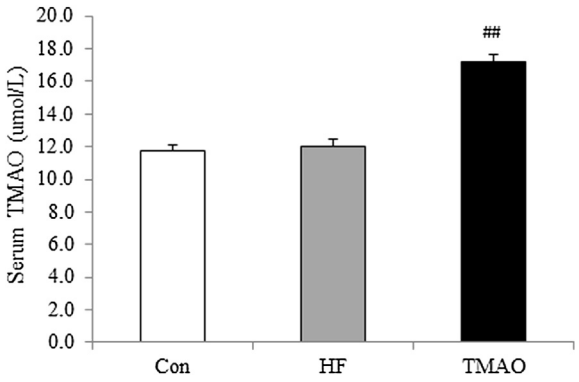

FIG. 1. Serum TMAO levels in C57BL/6 mice fed experimental diets. C57BL/6 mice feeding a normal diet, high fat diet, or high fat diet inducing 0.2% TMAO were sacrificed after 4 weeks. Serum TMAO levels were quantified by liquid chromatography with tandem mass spectrometry (LC/MS/MS). Data are presented as mean ± SEM; $n = 8$ mice per group. ##$P < 0.01$ compared to the HF group.

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