Chronic Feeding of a Nitric Oxide Synthase Inhibitor Induces Postprandial Hypertriglyceridemia in Type 2 Diabetic Model Rats, Otsuka Long-Evans Tokushima Fatty Rats, But Not in Nondiabetic Rats

Yutaka Nakaya, Noriko Ishimura, Eiko Takishita, and Akira Takahashi

Postprandial hyperlipidemia is frequently associated with diabetes mellitus and considered to be an independent coronary risk factor. Nitric oxide (NO) is a key regulator of glucose uptake in skeletal muscle. The goal of this study was to examine the effects of chronic in vivo competitive antagonism of NO synthase (NOS) by the administration of N$^{\omega}$-nitro-L-arginine methyl ester (L-NAME) on glucose and lipid metabolism in diabetic (Otsuka Long-Evans Tokushima Fatty [OLETF]) and nondiabetic rats. Chronic administration of L-NAME to rats induced reduced NO production and hypertension in both strains of rats. No detectable impairment of plasma levels of postprandial triglyceride (TG) or insulin sensitivity in nondiabetic rats was detected by chronic treatment of L-NAME, but significant impairment was observed in the cases of diabetic rats. These results suggest that diabetes, when associated with endothelial dysfunction, results in greater abnormalities in lipid, as well as glucose metabolism.

HYPERTRIGLYCERIDEMIA IS frequently associated with patients who have diabetes mellitus. Patients with type 2 diabetes mellitus are insulin resistant and exhibit postprandial lipid intolerance, even though they have normal fasting triglyceride (TG) levels.[1] Epidemiologic studies indicate that abnormal lipid metabolism, especially postprandial hypertriglyceridemia, is a powerful risk marker for atherosclerosis.[2] Hyperinsulinemia and insulin resistance are frequently associated with endothelial dysfunction. Endothelial dysfunction appears in the early stages of vascular abnormality, which is associated with hypertension,[3] diabetes,[4-7] hyperlipidemia,[8] and obesity.[9] Such endothelial abnormalities are associated with reduced activity of nitric oxide (NO). Our knowledge of the relationships between lipid and glucose metabolism and inhibition of NO synthesis is incomplete. In addition, recent studies indicate that hypertension could contribute to the increased risk of atherosclerosis in insulin-resistant diabetic subjects. The UK Prospective Diabetes Study (UKPDS)[10] showed that hypertension, when associated with type 2 diabetes mellitus, increases markedly the risk of microvascular complications, as well as macrovascular complications. The results of the Hypertension Optimum Treatment (HOT)[11] and Systolic Hypertension in Europe (Syst-Eur)[12] studies have clearly demonstrated that lowering blood pressure reduces stroke rate in both nondiabetic and diabetic patients, and that this benefit is significantly greater in diabetics.

To investigate the issue of whether endothelial dysfunction/hypertension, when associated with diabetes, synergistically worsens cardiovascular risk factors, the effect of chronic oral ingestion of an inhibitor of NO synthesis, N$^{\omega}$-nitro-L-arginine methyl ester (L-NAME), was examined with reference to lipid and glucose metabolism in diabetic model rats. Otsuka Long-Evans Tokushima Fatty (OLETF) rats, a model for type 2 diabetes, develop hyperglycemia, obesity, and insulin resistance, and as a result, serve as a model for human type 2 diabetes.[13] The results of our study showed that the chronic oral administration of L-NAME induces significant insulin resistance and postprandial hypertriglyceridemia in diabetic rats, but not in nondiabetic rats, although hypertension was observed in both types of rats treated with L-NAME.

From the Department of Nutrition, School of Medicine, The University of Tokushima, Tokushima City, Japan.

Submitted May 17, 2001; accepted December 7, 2001.

Address reprint requests to Yutaka Nakaya, MD, Department of Nutrition, School of Medicine, Tokushima University, 3-18-15, Kuramoto-cho, Tokushima City, 770-8503, Japan.

0026-0495/02/5106-0008$35.00/0

doi:10.1053/meta.2002.32726

MATERIALS AND METHODS

Animals and Experimental Design

The study protocol was reviewed and approved by the Committee on Ethics on Animal Experiments, the University of Tokushima, and the experiments were conducted according to the Guidelines for Animal Experiments of the University of Tokushima.

Male OLETF (diabetic) rats and nondiabetic control male Long-Evans Tokushima Otsuka (LETO) rats at 4 weeks of age were generously provided by the Tokushima Institute, Otsuka Pharmaceutical Co, Tokushima, Japan and were maintained in our animal facilities under specific pathogen-free conditions (Institute of Animal Experimentation, the University of Tokushima). The animals were individually housed in an air-conditioned room (23°C ± 1°C, lights on 8:00 AM to 8:00 PM). There were 6 groups of 10 rats each. Three groups were OLETF, and 3 were LETO. The 3 groups of each strain were: control diet, diet + L-NAME, diet + L-NAME + L-Arg. There were no rats that received control diet + L-Arg in the absence of L-NAME. At 11 weeks of age, rats were randomly assigned. Rats rats were fed diets (Type MF; Oriental Yeast, Tokyo, Japan) with or without 0.01% L-NAME (Sigma Chemical, St Louis, MO). The inhibition of L-NAME is competitive and reversible when a sufficient amount of L-arginine (L-Arg) was present. To study the effect of the addition of L-Arg, we added 1.4% L-Arg (Sigma) to rats supplemented with L-NAME (L-NAME+L-Arg group). The amount of food intake of the rats with or without treatment was similar. Previous studies involved the administration of L-NAME via drinking water. As the amount of drinking water was variable in each rat and much greater in OLETF rats than LETO rats, we added L-NAME to the food to adjust the amount of L-NAME/body weight. The daily amount of oral feeding of L-NAME was about 6 mg/kg for both OLETF and LETO rats.

Body weight and food intake were measured throughout the experimental period. After 13 weeks of age, blood pressure, blood levels of lipids, and glucose were measured. Oral lipid tolerance tests were performed at 13 weeks of age. At 16 weeks of age, a hyperinsulinemic

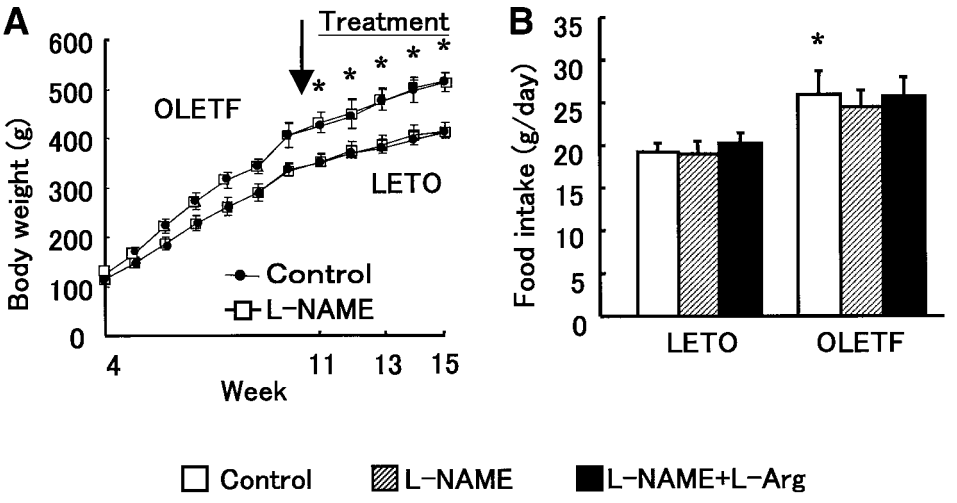

Fig 1. Change in weight (A) and food intake (B) of the experimental animals.

euglycemic clamp test was performed to evaluate peripheral insulin sensitivity. Following the completion of the euglycemic clamp test, the rats were killed, and the liver and intra-abdominal fat (mesenteric, epididymal, and retroperitoneal fat) were surgically removed and weighed separately.

Blood Pressure Measurements

At 13 weeks of age, blood pressure of the rats was measured by the tail-cuff methods using a noninvasive rat-mouse manometer transducer (TK-370C; Neuroscience, Tokyo, Japan). Measurements were taken at 3:00 PM, and the rats were warmed for 10 to 15 minutes at 28°C to make the pulsations of the tail artery detectable before the measurements. Values for systolic blood pressure were obtained by averaging readings from 5 continuous measurements.

Measurement of In Vivo Glucose Disposal by Hyperinsulinemic Euglycemic Clamp Studies

Insulin-mediated whole-body glucose uptake was determined in 16-week-old anesthetized rats using a hyperinsulinemic euglycemic clamp.[14] After an overnight fast, rats were anesthetized by an intraperitoneal injection of pentobarbital (50 mg/kg), and catheters (silicon tube) were inserted in the jugular veins and carotid arteries. The catheter was bathed in heparinized saline solution before insertion. Rats were then given an infusion of insulin (60 pmol/kg/min). A glucose solution (0.56 mol/L) was initiated at time 0, and the rate was adjusted to maintain the plasma concentration of glucose at approximately 6.1 mmol/L. The whole-body glucose uptake represents the glucose infusion rate (GIR) during the final 20 minutes.

Oral Lipid Tolerance Test

At 13 weeks of age, the rats underwent an oral lipid tolerance test after an overnight fast. Fresh cream (saturated fatty acid: 69.5%, Meiji Milk Products, Tokyo, Japan; 4 mL/kg body weight) was administered orally. Blood samples were obtained from a tail vein at 0, 1, 2, 4, 6, and 8 hours after the meal for measurement of plasma TG levels.

Biochemical Analysis

Plasma concentrations of TG were determined using the kits (Triglyceride G-Test Wako; Wako Pure Chemical, Osaka, Japan). The TG concentration of the liver was measured after extraction of lipids from liver tissue as described by Folch et al.[15] Plasma glucose levels were determined by the glucose oxidase method (Tido-Tidex; Sankyo, Tokyo, Japan). Insulin levels were determined by an enzyme-linked immunosorbent assay (ELISA) (Levis insulin rat; Shibayagi, Gunma, Japan) with rat insulin as the standard.

Urinary Nitrite and Nitrate Analysis

Both nitrite (NO_2^-) and nitrate (NO_3^-) are metabolites of NO. To estimate NO production, the urinary concentrations of NO_2^- and NO_3^- were measured. Twenty-four-hour urine samples were collected in bottles containing an antibiotic solution (1 mg/mL penicillin-G, 1 mg/mL streptomycin, and 0.25 mg/mL amphotericin-B) from 15-week-old rats. NO_3^- in urine was converted into NO_2^- with NO_3^- reductase and subsequently measured with the Griess reagent (Cayman Chemical Co, Ann Arbor, MI). The NO_2^- measured in this manner reflects the combination of NO_2^- and NO_3^- in the original sample. NO_2^- values were normalized by comparison to creatinine.

Statistical Analysis

Data in the text and figures are expressed as the means ± SD. Data were analyzed by analysis of variance (ANOVA) plus Bonferroni multiple comparison tests. $P < .05$ was assumed to be statistically significant.

RESULTS

Body Weight and Food Intake

The body weight gain and daily food intake during the experimental period for diabetic (OLETF) rats were greater than those for nondiabetic (LETO) rats (Fig 1), but there were no significant differences between the groups with and without feeding of L-NAME.

Urinary NO_2^- + NO_3^- Excretion Rate and Blood Pressure

Urinary NO_2^- + NO_3^- excretion rate, normalized by urinary creatinine, for OLETF rats was similar to that of LETO rats (Fig 2A). Urinary NO_2^-+ NO_3^- excretion was significantly decreased in both LETO and OLETF rats treated with L-NAME. Decreased urinary NO_2^- + NO_3^- excretion as the result of L-NAME treatment was significantly reversed by the addition of excess L-Arg.

The systolic blood pressure of OLETF rats was significantly

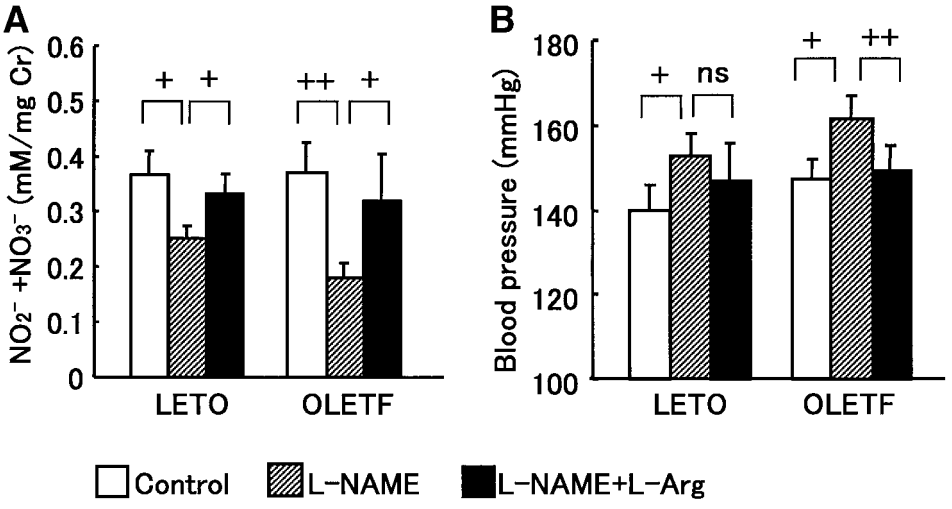

Fig 2. Effect of chronic oral ingestion of L-NAME or L-NAME + L-Arg on normalized urinary NO_2^- + NO_3^- excretion rate (A) and systolic blood pressure (B) in LETO and OLETF rats. Data are expressed as means ± SD (n = 6 to 10 in each group). $^+P < .05$, $^{++}P < .01$.

greater than that of LETO rats (Fig 2B). The blood pressure of the L-NAME-treated rats was higher than that for the non-treated OLETF and LETO rats. The elevation in systolic blood pressure by L-NAME was significantly suppressed by the addition of L-Arg in OLETF rats, but not significantly in LETO rats. Diastolic blood pressure, however, was significantly revered by the addition of L-Arg in LETO rats (control, 89 ± 5 mm Hg; L-NAME, 110 ± 5 mm Hg; L-NAME+L-Arg, 89 ± 13 mm Hg).

Plasma Glucose, Insulin, and Lipids

Fasting plasma levels of glucose and insulin in OLETF rats were significantly higher than in LETO rats (Fig 3A). Fasting plasma glucose levels were not altered by treatment with L-NAME or L-NAME+L-Arg in both types of rats, respectively. Fasting plasma insulin levels were significantly increased in L-NAME-treated OLETF rats, but the addition of L-Arg did not suppress elevation in plasma insulin levels (Fig 3B). There was no significant effect of L-NAME with and without L-Arg on plasma insulin levels in LETO rats.

The GIR of OLETF rats was significantly decreased compared with that of LETO rats (Fig 4A). GIR was significantly decreased in OLETF rats treated with L-NAME, and this decrease was reversed in OLETF rats that were treated with additional L-Arg. However, the changes were smaller, and the difference did not reach the level of statistical significance in LETO rats.

Liver concentrations of TG were also increased in OLETF rats compared with that of LETO rats (Fig 4B). They were increased by L-NAME treatment in OLETF rats, but not in LETO rats. The liver concentration of TG was significantly correlated with GIR ($r = -0.789$, $P < .01$).

Oral Lipid Tolerance Test

An oral lipid tolerance test showed that compared with LETO rats, plasma concentrations of TG at each time point

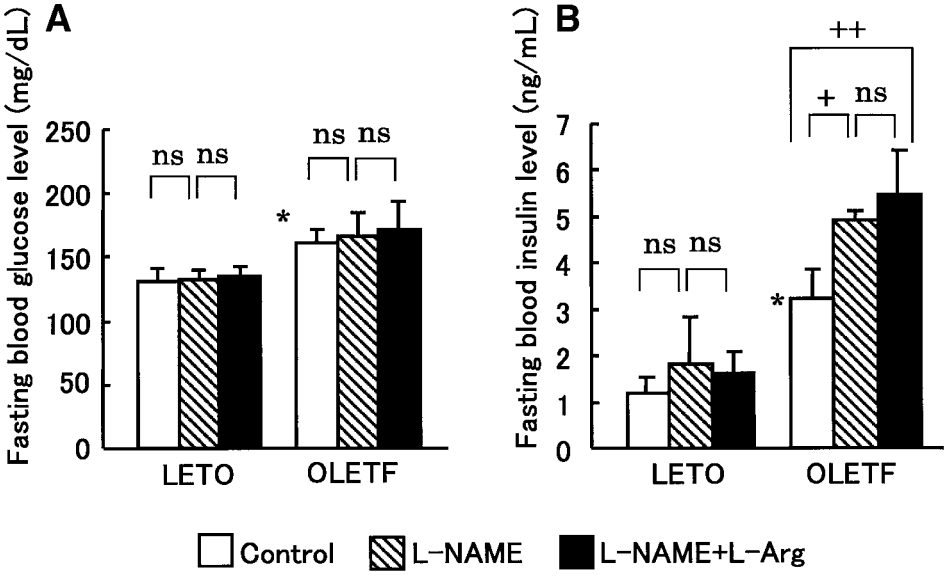

Fig 3. Effect of chronic oral ingestion of L-NAME and L-NAME + L-Arg on fasting blood levels of (A) glucose and (B) insulin. OLETF rats show higher blood glucose and insulin levels. There is no significant difference in fasting blood glucose levels among groups with and without treatment of L-NAME and L-Arg. In OLETF rats, fasting insulin levels were increased by treatment with L-NAME, but addition of L-arg did not reverse it. *$P < .05$ OLETF *v* LETO, $^+P < .05$, $^{++}P < .01$ between 2 groups indicated by the bar.

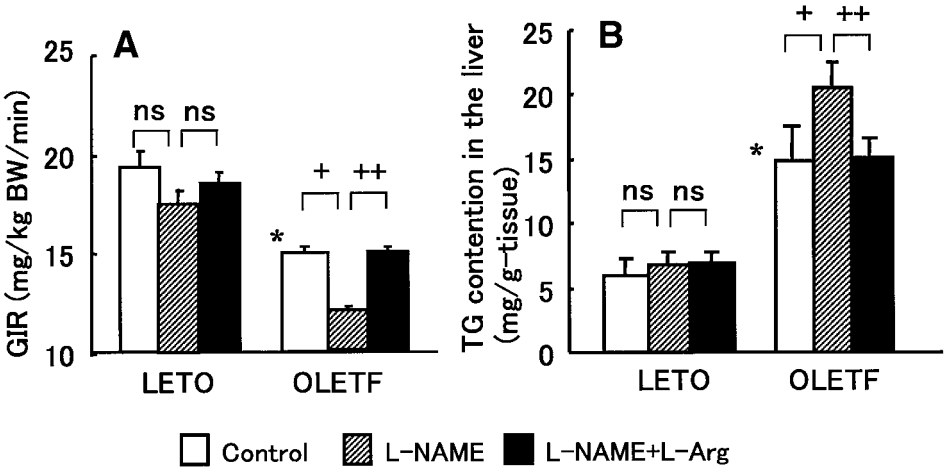

Fig 4. (A) Effect of chronic oral ingestion of L-NAME or L-NAME + L-Arg on GIR in OLETF rats. (B) Liver concentration of TG. Data are expressed as means ± SD (n = 8 to 10 in each group). *P < .01 OLETF *v* LETO, ^{+}P = .05, ^{++}P < .01.

were significantly increased in OLETF rats, and appearance of its peak was delayed (Fig 5A). The elevation of TG levels persisted for over 4 hours in OLETF rats. In the case of OLETF rats treated with L-NAME, plasma TG levels were further elevated at 4 hours, and these higher levels persisted at 6 and 8 hours after lipid loading in OLETF rats. In LETO rats, there was no significant difference in postprandial plasma TG levels between those with and without L-NAME administration, and the 2 curves are nearly superimposable. Figure 5B shows the area under curves of TG after lipid loading in each group. A significant effect of L-NAME treatment was observed in OLETF rats only. The elevated postprandial blood levels of TG were reversed by addition of L-Arg to the L-NAME-treated OLETF rats.

DISCUSSION

We demonstrate herein that the chronic inhibition of NO synthesis by L-NAME induces postprandial hyperglycemia in OLETF rats, insulin-resistant diabetic model animals, but not in nondiabetic LETO rats. Insulin resistance was significantly increased in OLETF rats only. These changes were reversed by the addition of an excess of L-Arg. The results indicate that diabetes, when associated with endothelial dysfunction, further enhances insulin resistance and hyperlipidemia, which are risk factors for atherosclerosis.

NO, which is synthesized in the vascular endothelium, is a major mediator of endothelium-derived relaxing factor.[16] In addition to its role in controlling vascular tone and platelet aggregation, evidence suggests that NO enhances the action of insulin, thus augmenting glucose uptake by skeletal muscles and adipose tissues.[17] Insulin increases glucose transport, in part, by increasing blood flow and glucose delivery to the muscle, a process that is mediated by the release of NO from the endothelium.[18] Exogenously administered NO, which is generated from the NO donor, sodium nitroprusside (SNP), stimulates glucose transport in isolated skeletal muscles[19-21] by increasing GLUT4 levels at the cell surface.[22]

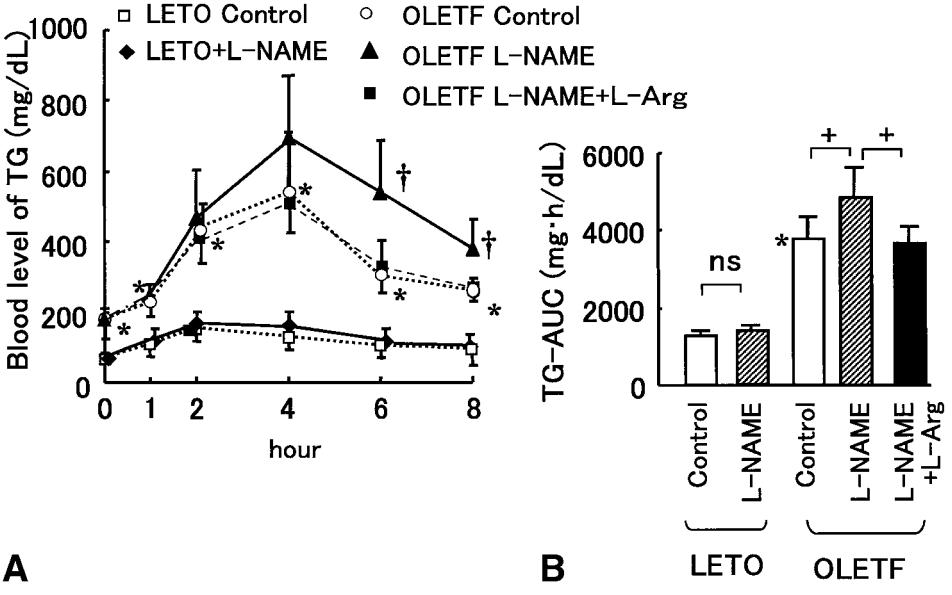

Fig 5. Change in the plasma level of TG in response to an oral lipid tolerance test (A) and a comparison between areas under the curve (AUC) of TG (B) by chronic oral ingestion of L-NAME or L-NAME + L-Arg in OLETF rats. Data are expressed as means ± SD (n =7 to 8 in each group). *P < .01, OLETF *v* LETO, ^{+}P < .05 L-NAME treatment *v* control.

There have been, however, few studies reported on lipid metabolism by NO, especially postprandial hypertriglyceridemia. The acute administration of the NO synthase (NOS) inhibitors, N^{ω}-monomethyl-L-arginine (L-NMMA) or L-NAME results in the development of insulin resistance, hypertension, and/or hyperglycemia.[17,18] In contrast to the effects of inhibiting NO synthesis in vivo, NOS inhibitors fail to affect insulin-stimulated glucose transport in isolated muscles incubated using in vitro preparations,[17,20,22] although the inhibition of NO synthesis decreases blood flow to skeletal muscle and impairs insulin-mediated glucose disposal during a hyperinsulinemic euglycemic clamp in vivo. These results suggest that hemodynamic factors are needed to fully amplify the increase in insulin-stimulated glucose transport in skeletal muscle.

Different from these studies, Swislocki et al[23] showed, in in vivo studies, that the chronic feeding of L-NAME caused hypertension, but not insulin resistance. These results suggest that the contradictory results are due to the amount of inhibitor and differences in the model animals used. In the present study, nondiabetic rats treated with L-NAME showed hypertension, but no significant change in fasting blood glucose levels or postprandial levels of TG. Changes in other parameters, such as GIR, and fasting blood levels of insulin were also greater in OLETF rats than in LETO rats. The mechanism for why a blockade of NO synthesis had a greater influence in diabetic rats than in nondiabetic rats was not investigated in the present study. However, these results are consistent with the higher incidence of cardiovascular complications observed in diabetic patients with hypertension.

Several studies showed that hypertriglyceridemia is a risk factor for cardiovascular disease.[24-26] Increased postprandial TG levels may result from an excess in the production of very–low-density lipoprotein (VLDL) by the liver or a reduced clearance of TG. The possibility of a decrease in the clearance of TG-rich lipoprotein cannot be excluded. Lipoprotein lipase (LPL) is a rate-limiting enzyme for clearance. LPL is strongly modulated by the nature and amount of substrate, and its activity in each tissue differs widely. Therefore, it is difficult to determine total LPL activity in vivo. On the other hand, the fact that apoprotein-B–containing particles were increased in diabetic patients suggests that the vast majority of postprandial hypertriglyceridemia is due to an increased production of VLDL.[27] Thus, one likely mechanism for postprandial hypertriglyceridemia is that insulin resistance in either the liver or adipose tissue (or both) fails to curtail VLDL output during the postprandial period. The increased production of VLDL is closely related to increased intrahepatocyte TG stores.[28] In agreement with these findings, we also found an increase in TG levels in the liver in L-NAME-treated rats.

There have been few studies of NO effect on lipid metabolism. Khedara et al[29] found that L-N^{ω} nitroarginine (L-NNA), another inhibitor of NO synthase, inhibited carnitine palmitoyltransferase, the rate-limiting enzyme of fatty acid oxidation, but did not affect fatty acid synthase, which are lipogenic enzymes. These results indicate that inhibition of NOS elevated serum TG levels by lowering hepatic fatty acid oxidation. L-Arg is used as a dietary supplement, presumably to enhance endothelial function. There exists a small amount of endogenous inhibitors of NO synthesis, such as N^{ω}-monomethylarginine (MMA) and NG, N^{ω}-dimethylarginine (DMA), in the human body. Our results suggest the possibility that supplementation of a small dose of L-Arg could improve hypertriglyceridemia.

Tight blood pressure control in patients with hypertension and type 2 diabetes achieves a clinically important reduction in the risk of deaths related to diabetes, complications related to diabetes, the progression of diabetic retinopathy, and a deterioration in visual acuity.[10] This study supports the hypothesis that the association with hypertension/endothelial dysfunction and diabetes may worsen lipid metabolism and insulin resistance, which are major risk factors in atherosclerosis.

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