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Feeding the nitric oxide synthase inhibitor L-N$^{\omega}$nitroarginine elevates serum very low density lipoprotein and hepatic triglyceride synthesis in rats

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This study was conducted to study the influence of dietary L-N$^{\omega}$nitroarginine (L-NNA), a nitric oxide (NO) synthase inhibitor, on serum lipids and lipoproteins and on the activities of enzymes related to lipid metabolism in rats. Feeding rats a diet containing 0.2 g/kg L-NNA for 5 weeks elevated serum concentrations of triglyceride, cholesterol, phospholipid, and free fatty acid and reduced serum nitrate (an oxidation product of NO). The elevation in serum triglyceride was mainly due to the elevation in very low density lipoprotein (VLDL) triglyceride. Contents of cholesterol and phospholipid in the VLDL fraction also were elevated by L-NNA. L-NNA treatment caused significantly higher activity of hepatic microsomal phosphatidate phosphohydrolase (the rate-limiting enzyme in triglyceride synthesis) and lower activity of hepatic carnitine palmitoyltransferase (the rate-limiting enzyme in fatty acid oxidation). Activities of hepatic enzymes responsible for fatty acid synthesis such as glucose-6-phosphate dehydrogenase, malic enzyme, and fatty acid synthase were unaffected by L-NNA. The activity of hepatic microsomal phosphocholine cytidyltransferase (the rate-limiting enzyme in phosphatidylcholine synthesis) was reduced significantly by L-NNA. Our results suggest that lower NO production caused the elevations in hepatic triglyceride synthesis by higher esterification of fatty acid and lower fatty acid oxidation, leading to an enrichment of VLDL triglyceride. (J. Nutr. Biochem. 10:274–278, 1999)

Keywords: nitric oxide; serum lipoproteins; hypertriglyceridemia; phosphatidate phosphohydrolase; carnitine palmitoyltransferase

Introduction

Nitric oxide (NO) is an important cellular regulator.[1,2] It has been shown to play roles in blood vessel dilation,[1,2] immune reactions,[1,3] and the central and peripheral nervous systems.[1,2] NO production is enhanced by estrogen, inflammation, and exercise through elevation of NO synthase activity.[4–8] NO is inactivated by reaction with superoxide anion,[1] and oxidative stress causes lower level of NO, which in turn causes some aggravation effects such as hypertension.[9]

Recently we have found that feeding L-N$^{\omega}$nitroarginine (L-NNA), which is a powerful specific inhibitor of NO synthase, to rats caused higher concentrations of serum triglyceride and cholesterol and lower serum nitrate (an oxidation product of NO).[10] Adding excess L-arginine to the diet containing L-NNA elevated serum nitrate by suppressing competitive inhibition of NO synthase by L-NNA, and suppressed elevations of these lipids in serum. On the basis of these facts, we speculate that lower NO production causes hyperlipidemia.[10] Kurowska and Carrol[11] also reported that feeding rabbits a diet containing the NO donor sodium nitroprusside caused a reduction in low density lipoprotein (LDL) cholesterol and a trend of reduction in

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serum total cholesterol. Local generation of NO within the epicardial coronary arteries serves to inhibit platelet adhesion and aggregation[12] and to inhibit smooth muscle proliferation.[13] Therefore, lower NO generation seems to lead to atherosclerosis.

Our previous study provided evidence that hypercholesterolemia caused by L-NNA is mediated by lower synthesis of bile acid from cholesterol,[14] and that hypertriglyceridemia caused by L-NNA is due in part to lower hepatic fatty acid oxidation.[10] In this study, we further examined the influence of L-NNA on serum lipoproteins and on hepatic enzymes related to triglyceride synthesis in rats.

Materials and methods

Animals and diets

Male Wistar rats (Hiroshima Laboratory Animal Center, Hiroshima, Japan) weighing 50 to 70 g were used. Animals were individually housed in metal cages in a temperature-controlled (24°C) room with a 12-hour light-dark cycle (lights on, 8:00 AM to 8:00 PM). All rats had free access to deionized water and experimental diet. Composition of the basal diet was (in g/kg): casein, 200; sucrose, 217; α-corn starch, 433; corn oil, 50; cellulose powder, 50; salt mixture,[15] 35; vitamin mixture,[15] 10; DL-methionine, 3; and choline bitartrate, 2. L-NNA (Aldrich Chemical Company Inc., Milwaukee, WI USA) was added to the basal diet at the level of 0.2 g/kg. After 5 weeks of consuming the diets, food was removed from the cages at 8:00 AM, and the rats were lightly anesthetized with diethylether and euthanized between 1:00 PM and 3:00 PM. Blood was collected by heart puncture, and samples were allowed to clot on ice. Serum samples were obtained by centrifugation. Liver was immediately removed, weighed, and stored at −80°C until use. Portions of the fresh liver were used for preparation of subcellular fractions.

Analytical procedures

Serum lipoprotein fractions [very low density lipoprotein (VLDL), d < 1.006 g/mL; LDL, d:1.006–1.063 g/mL; and high density lipoprotein (HDL), d:1.063–1.210 g/mL] were separated by stepwise density-gradient ultracentrifugation (TL-100, Beckman, San Francisco, CA USA).[16] Total liver lipids were extracted by the method of Folch et al.[17] Concentrations of triglyceride, cholesterol, phospholipid, and free fatty acid were measured by kits from Wako Pure Chemical Co. (Osaka, Japan). Concentration of ketone bodies (acetoacetate and 3-hydroxybutyrate) were measured by a kit (Ketone Test Sanwa Chemical Institute, Nagoya, Japan). Concentrations of serum apolipoproteins (apo A-I, A-IV, B, and E) were estimated by rocket electroimmunoassay.[16] To estimate NO production, serum concentration of nitrate (an oxidation product of NO) was measured by a kit (Nitrate/Nitrite Assay Kit, Cayman Chemical Co. Ann Arbor, MI USA).

Portions of the fresh liver from individual rats were homogenized in an ice-cooled 0.25 M sucrose solution containing a 10 mM Tris-HCl buffer (pH 7.4) and 1 mM EDTA. Microsomal and cytosolic fractions were prepared as described previously.[18] The fractions were stored at −80°C. Protein was assayed by a kit (Bio-Rad Protein Assay, Bio-Rad Laboratories, Richmond, CA USA) using bovine serum albumin as the standard.

Activity of Mg^{2+}-dependent phosphatidate phosphohydrolase (PAP) in liver microsomes and cytosol was assayed as described previously.[18] Activities of phosphocholine cytidyltransferase (CTP) in the microsomes and cytosol and of choline kinase (CK) in the cytosol were measured by the reported methods.[19,20] Activities of glucose-6-phosphate dehydrogenase (G6PD), malic enzyme (ME), and fatty acid synthase (FAS) in the cytosol were assayed spectrophotometrically as described by Freedland[21] and Martin et al.,[22] respectively. Activity of hepatic carnitine palmitoyltransferase (CPT) in liver homogenate was measured using L-carnitine, palmitoyl CoA, and 5,5′-dithio-bis (2-nitrobenzoic acid) according to the method of Bieber and Fiol.[23]

Results were expressed as means ± SE and analyzed by Student's *t*-test.

Table 1. Effect of dietary L-NNA on serum lipids and apolipoproteins in rats

	Control	L-NNA
Serum triglyceride (mmol/L)	2.01 ± 0.28	3.45 ± 0.40[a]
Serum cholesterol (mmol/L)	4.37 ± 0.24	5.15 ± 0.24[a]
Serum phospholipid (mmol/L)	2.75 ± 0.12	3.12 ± 0.10[a]
Serum free fatty acid (mEq/L)	385 ± 25	488 ± 30[a]
Serum ketone bodies (μmol/L)	255 ± 25	235 ± 27
Serum nitrate (μmol/L)	59 ± 5	28 ± 2[a]
Apo A-I (mg/L)	656 ± 30	773 ± 34[a]
Apo A-IV (mg/L)	179 ± 3	211 ± 6[a]
Apo B (mg/L)	77 ± 4	86 ± 5
Apo E (mg/L)	527 ± 15	557 ± 18
Apo B/apo A-I	0.121 ± 0.010	0.112 ± 0.006

Mean ± SE ($n = 10$).
[a]Significantly different from the contorl group ($P < 0.05$).
L-NNA–L-N$^{\omega}$ nitroarginine.

Results

Gain in body weight (g/5 wk) was unaffected by L-NNA feeding ($P > 0.05$; control 285 ± 4, L-NNA 270 ± 6). Food intake (g/5 wk) also was unaffected by L-NNA ($P > 0.05$; control 712 ± 14, L-NNA 685 ± 18).

Serum concentrations of triglyceride, cholesterol, and phospholipid were higher in the L-NNA group than in the control group ($P < 0.05$; *Table 1*). Serum free fatty acid was significantly elevated in the L-NNA group, whereas serum ketone bodies were unaffected by L-NNA. Serum concentration of nitrate was significantly reduced by L-NNA.

Serum concentrations of apo A-I and A-IV were significantly higher in the L-NNA group than the control group, whereas concentrations of apo B and E were unaffected by L-NNA. The ratio of apo B:apo A-I was unaffected by L-NNA.

Concentrations of triglyceride in the VLDL, LDL, and HDL fractions were significantly higher in the L-NNA group than in the control group ($P < 0.05$; *Figure 1*). Elevation in serum triglyceride by L-NNA treatment was due mainly to the elevation in VLDL triglyceride. L-NNA feeding also elevated VLDL cholesterol ($P < 0.05$), whereas concentrations of cholesterol in the LDL and VLDL fractions were unaffected by L-NNA. Concentrations of phospholipid in the VLDL and HDL fractions were elevated by L-NNA ($P < 0.05$). Concentration of LDL phospholipid was unaffected by L-NNA.

Relative liver weight and concentrations of hepatic cholesterol and phospholipid were unaffected by L-NNA (*Table 2*; $P > 0.05$). There was a trend of elevation in liver triglyceride concentration in rats that received L-NNA ($0.05 < P < 0.1$). Activities of G6PD, ME, and FAS were

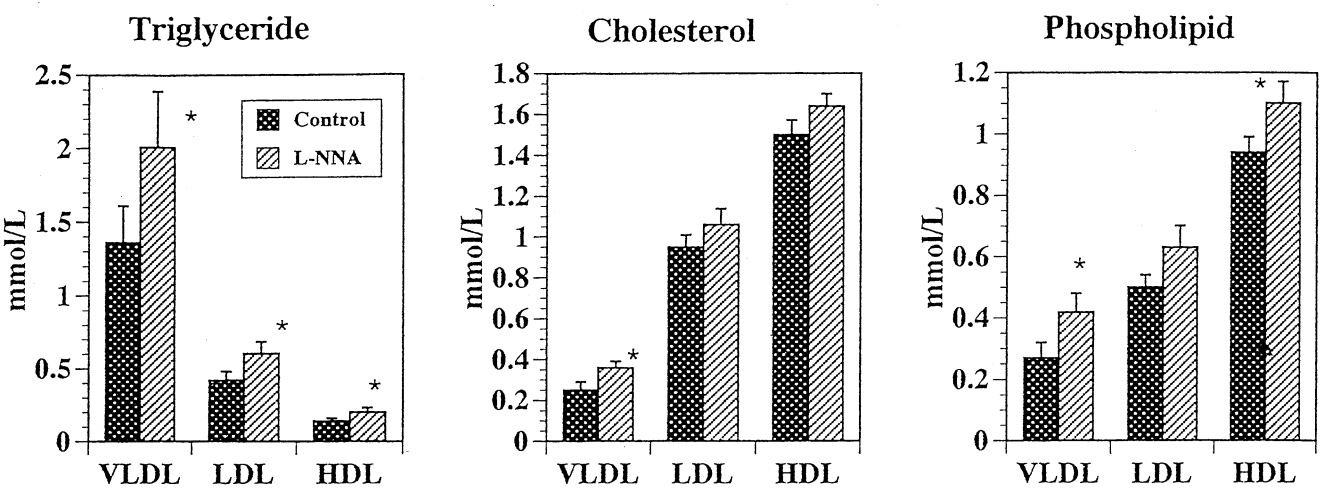

Figure 1 Distributions of triglyceride, cholesterol, and phospholipid among various lipoprotein fractions in rats fed diet with or without L-N$^{\omega}$nitroarginine (L-NNA). The vertical bars indicate the SE ($n = 10$). *$P < 0.05$. VLDL, very low density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein.

unaffected by L-NNA. Activity of CPT, which is the rate-limiting enzyme of mitochondrial β-oxidation, was reduced significantly by L-NNA addition.

The activity of microsomal Mg^{2+}-dependent PAP, which controls the branching point in glycerolipid biosynthesis, was elevated significantly by L-NNA, whereas the cytosolic activity was unaffected (*Table 3*). Microsomal activity of CTP, the rate-limiting enzyme in phosphatidylcholine biosynthesis, was reduced significantly by L-NNA, whereas the cytosolic activity was unaffected. Cytosolic activity of CK, the first enzyme on the de novo phosphatidylcholine biosynthesis pathway, was unaffected by L-NNA.

Discussion

Consistent with our previous study[10] was the finding that L-NNA treatment caused a marked hypertriglyceridemia. On the other hand, elevations in serum cholesterol and phospholipid by L-NNA were only slight. The present study further demonstrated that the hypertriglyceridemia by L-NNA was ascribed mainly to a higher concentration of triglyceride in VLDL fraction. Concentrations of cholesterol and phospholipid in VLDL fraction also were clearly elevated by L-NNA, but not by very much. NO appears to be an important regulator of serum VLDL triglyceride.

L-NNA treatment caused higher serum free fatty acid and lower activity of hepatic CPT (the rate-limiting enzyme of fatty acid oxidation) without affecting hepatic activities of G6PD, ME, and FAS. This study further demonstrated higher activity of PAP and lower activity of CTP in liver microsomes by L-NNA. It has been suggested that PAP and CTP are involved in the rate-limiting step of triglyceride synthesis and phosphatidylcholine synthesis, respectively, and appear to exist in both soluble and particle forms, with the distribution of these forms being affected by the prevailing metabolic status.[19,24–26] The enzymes translocate from cytosol to the endoplasmic reticulum to become functionally active and may help to regulate glycerolipid and phospholipid metabolisms.[19,25] All of these results suggest that dietary L-NNA causes higher triglyceride

Table 2. Effect of dietary L-NNA on liver lipids and the activities of enzymes relating to fatty acid synthesis and oxidation in rats

	Control	L-NNA
Liver weight (g/kg of body wt)	40.3 ± 0.7	41.1 ± 1.1
Liver triglyceride (μmol/g tissue)	14.6 ± 2.0	29.9 ± 3.8
Liver cholesterol (μmol/g tissue)	6.96 ± 0.14	6.88 ± 0.19
Liver phospholipid (μmol/g tissue)	23.2 ± 0.6	22.8 ± 0.5
Glucose-6-phosphate dehydrogenase (nmol/min mg of cytosol protein)	95 ± 9	100 ± 13
Malic enzyme (nmol/min · mg of cytosol protein)	91 ± 6	92 ± 8
Fatty acid synthase (nmol/min · mg of cytosol protein)	5.2 ± 0.2	5.1 ± 0.3
Carnitine palmitoyltransferase (nmol/min · mg of liver protein)	4.7 ± 0.2	3.4 ± 0.2[a]

Mean ± SE ($n = 10$).
[a]Significantly different from the control group ($P < 0.05$).
L-NNA–L-N$^{\omega}$ nitroarginine.

Table 3. Effect of dietary L-NNA on the activities of hepatic phosphatidate phosphohydrolase, phosphocholine cytidyltransferase, and choline kinase in rats

	Control	L-NNA
Phosphatidate phosphohydrolase		
Microsomes (nmol/min · mg of protein)	16.8 ± 0.7	19.8 ± 0.9[a]
Cytosol (nmol/min · mg of protein)	19.9 ± 0.4	20.4 ± 0.8
Phosphocholine cytidyltransferase		
Microsomes (nmol/min · mg of protein)	1.88 ± 0.12	1.52 ± 0.03[a]
Cytosol (nmol/min · mg of protein)	0.65 ± 0.03	0.72 ± 0.05
Choline kinase		
Cytosol (nmol/min · mg of protein)	2.25 ± 0.16	2.02 ± 0.27

L-NNA–L-N$^{\omega}$ nitroarginine.

synthesis by increasing esterification of fatty acid and lower hepatic fatty acid oxidation, leading to the elevations of serum and VLDL triglyceride. On the other hand, the lower phosphatidylcholine synthesis also may indirectly enhance the synthesis of triglyceride by increasing fatty acids available for triglyceride synthesis. This assumption explains why the elevation of serum triglyceride by L-NNA was more prominent than that in serum phospholipid.

Recently we demonstrated that hypercholesterolemia caused by the inhibition of NO synthesis was at least in part mediated through lower activity of cholesterol 7 α-hydroxylase, the rate-limiting enzyme of bile acid synthesis from cholesterol.[14] Activities of hepatic cholesterol 7 α-hydroxylase and CPT have been suggested to be regulated by protein kinase C.[27,28] This indicates that higher activity of protein kinase C may cause lower activities of both cholesterol 7 α-hydroxylase and CPT.[27,28] It also has been reported that acylation stimulating protein acts to stimulate triglyceride synthesis by increasing the rate of esterification of fatty acid via activation of protein kinase C.[29] On the other hand, NO has been reported to inhibit the activity of protein kinase C.[30] Therefore, we postulate that lower NO level might cause higher activity of hepatic protein kinase C, which in turn results in lower activities of cholesterol 7 α-hydroxylase and CPT and in higher activity of PAP, leading to hyperlipidemia. Further study is in progress to test this hypothesis.

Because apo B and E are main constituents of VLDL, we expected that L-NNA treatment might elevate serum apo B and E. However, this possibility was eliminated by no significant response of serum apo B and E to L-NNA treatment. Synthesis and/or secretion of these apolipoproteins might not be involved in the effect of L-NNA on serum VLDL.

Interestingly, L-NNA feeding resulted in significant elevations in serum apo A-I and A-IV (*Table 1*). Higher levels of apo A-I and A-IV seem to relate to elevations in HDL triglyceride and phospholipid because these apolipoproteins are major components of HDL. Recently, higher plasma apo A-IV level has been reported in hypertriglyceridemic patients.[31] In addition, apo A-IV has been reported to enhance the activation of lipoprotein lipase by apo C-II, which suggests a role in the metabolism of triglyceride-rich lipoproteins.[32] Further study is in progress to examine the influence of L-NNA treatment on the activity of lipoprotein lipase or the utilization of serum triglyceride in adipose tissues and muscles.

A recent study by Minami et al.[33] demonstrated that triglyceride-rich human plasma suppressed the NO synthesis in human endothelial cells. Together with our study, lower NO appears to induce hypertriglyceridemia, which in turn causes further suppression of NO production, leading to exaggeration of some aggravation effects such as hypertension associated with lower NO.

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