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Diet-Induced Protection against Lipopolysaccharide Includes Increased Hepatic NO Production[1]

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The host response to Gram-negative infection includes the elaboration of numerous proinflammatory agents, including tumor necrosis factor α (TNFα) and nitric oxide (NO). A component of the hepatic response to infection is an elevation in serum lipids, the so-called "lipemia of sepsis," which results from the increased production of triglyceride (TG)-rich lipoproteins by the liver. We have postulated that these lipoproteins are components of a nonadaptive, innate immune response to endotoxin [lipopolysaccharide (LPS)] and have previously demonstrated the capacity of TG-rich lipoproteins to protect against endotoxicity in rodent models of sepsis. Herein we report the capacity of a high-fructose diet to protect against LPS, most likely by inducing high circulating levels of endogenous TG-rich lipoproteins. The protective phenotype included the increased production of NO by hepatic endothelial cells. Rats, made hypertriglyceridemic by fructose feeding, experienced decreased LPS-induced mortality ($P < 0.03$) and systemic TNFα levels ($P < 0.05$) as compared with normolipidemic (chow-fed) controls. The increased survival was associated with elevated levels of inducible NO synthase (NOS2) mRNA levels and NO production (82 ± 26 vs 3 ± 3 nmol nitrite/10^6 cells, $P < 0.001$) by hepatic endothelial cells. Nonselective NOS inhibitors reversed the protective phenotype *in vivo* and readily decreased NO production by cultured endothelial cells from hypertriglyceridemic rats *in vitro.* This study suggests that a high-fructose diet can protect against endotoxicity in part through induction of endogenous TG-rich lipoproteins and hepatic endothelial cell NO production. This is the first report of diet-induced hyperlipoproteinemia and subsequent protection against endotoxemia.

Key Words: **hypertriglyceridemia; lipoproteins; endotoxin; endothelial cells; liver; sepsis; very low density lipoprotein.**

INTRODUCTION

Gram-negative sepsis is initiated by exposure to endotoxin [lipopolysaccharide (LPS)], a structural component of the bacterial cell wall. In reaction to LPS the host activates both pro- and anti-inflammatory responses, including the production of cytokines [e.g., tumor necrosis factor α (TNFα)] and acute-phase reactants, respectively. A component of the acute-phase response is an elevation in serum lipids, the so-called "lipemia of sepsis," which results from the increased production of triglyceride (TG)-rich lipoproteins by the liver [1–6]. Lipoproteins [e.g., chylomicrons and very low density lipoprotein (VLDL)] protect against endotoxicity by binding LPS to form lipoprotein–LPS complexes that are rapidly removed from plasma by the liver [7–10]. Therefore, we have postulated that TG-rich lipoproteins are part of a nonadaptive, innate immune response to infection [7, 11–13]. Accordingly, during Gram-negative sepsis hepatic lipoproteins are released into the circulation, serving to scavenge for and neutralize LPS and, thus, ameliorate the effects of this toxic macromolecule.

We have previously demonstrated the capacity of chylomicrons to protect against endotoxicity in several rodent models of sepsis; however, these experiments

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routinely involved the administration of exogenous lipoproteins that were preincubated with LPS to facilitate lipoprotein–LPS complex formation. While these reports confirm the concept that TG-rich lipoproteins can protect against LPS *in vivo*, the therapeutic implications of this observation are uncertain. To demonstrate that a lipid-based therapy for sepsis could be clinically useful the infused lipid must effectively bind and neutralize circulating LPS *in vivo*, without the *ex vivo* preincubation step employed in the earlier studies [7, 11–14]. Herein we report the association between diet-induced hyperlipoproteinemia and the protection of rats against endotoxemia.

METHODS

Reagents and solutions. Apyrogenic, preservative-free 0.9% NaCl and H_2O (Abbott Laboratories, North Chicago, IL); corticosterone and D-galactosamine hydrochloride (Sigma Chemical Co., St. Louis, MO); glucose, 6% hydrogen peroxide, Hepes, and Nycodenz (Fisher Scientific, Fair Lawn, NJ); L-15 salts solutions, Ham's F12 medium, Dulbecco's modified Eagle's medium (DMEM), medium 199 OR, fetal calf and horse sera, and T2 RNase (Gibco BRL, Gaithersburg, MD); penicillin G (Marsam Pharmaceuticals, Inc., Cherry Hill, NJ); gentamicin sulfate (Lyphomed, Deerfield, IL); collagenase B, Pronase, and DNase I (Boehringer-Mannheim, Indianapolis, IN); heparin (Upjohn Co., Kalamazoo, MI); aminoguanidine HCl, N^G-nitro-L-arginine methyl ester (L-NAME), and N^G-nitro-L-arginine (L-NNA, Toris Cookson, St. Louis, MO) were used as specified.

Endotoxin. *Escherichia coli*, strain 055:B5 LPS (Sigma), was reconstituted with sterile, apyrogenic saline to a concentration of 100 μg/ml and stored in 0.5-ml aliquots at −70°C. This preparation of LPS was isolated via the Westphal technique (phenol extraction) [15], and had a specific activity of 6 endotoxin units (EU)/ng (USP reference LPS).

Depyrogenation. To avoid contamination with exogenously derived LPS, all heat-stable materials used in the isolation, processing, and assay of solutions to be injected into the rats were rendered sterile and free of detectable LPS ($\leq$ 5–10 pg/ml) by previously reported methods [12, 16, 17].

Animals. Sprague–Dawley rats (male, 250–350 g) were purchased from Simonsen Animal Laboratories (Gilroy, CA). The animals were maintained under a controlled 12-h light cycle and provided either standard rat chow or a high-fructose semisynthetic diet (60% fructose, 20.7% casein, 8% cellulose, 5% lard; Harlan Teklad, Madison, WI) and water *ad libitum* for at least 2 weeks before conducting all experiments. All procedures were performed in accordance with institutional guidelines for the care and use of laboratory animals.

Lethality studies. Rats were infused with a LD_{70} dose of LPS (20 μg/kg) plus D-galactosamine (375 mg/kg) via the ileofemoral vein. Mortality was determined over a 48-h period during which food and water were provided *ad libitum*. In additional studies, animals received an intravenous injection of a NO synthase inhibitor 3 h after LPS infusion and mortality was determined at 48 h.

Lipid and TNFα assays. Total plasma triglyceride and cholesterol concentrations were determined using commercial, enzymatic assay kits (Wako, Wako, TX). Cell culture supernatants were analyzed for TNFα content using a commercial sandwich-style ELISA kit (Genzyme Immunobiologicals, Cambridge, MA), according to the manufacturer's instructions.

Liver cell isolation. All cells were isolated 60 min following the injection of LPS. Hepatocytes were isolated from rats as described [18]. Briefly, the portal vein was cannulated with a 16-gauge catheter. After collection of 3–4 ml of heparinized whole blood, the liver was perfused with warm, calcium-free buffer at the rate of 16 ml/min for several minutes. Once the liver was visibly cleared of blood, the perfusate was changed to 0.013% collagenase solution for approximately 20 min. The liver was then excised, minced, and placed in a rotary water bath with 0.01% DNase at 37°C for 30 min. The liver suspension was then filtered through a single layer of sterile cotton gauze and the hepatocytes were sequentially washed with Ham's/DMEM buffer solution containing 0.0005% DNase. Isolated hepatocytes were further purified by centrifugal elutriation [19]. Purity was routinely >99% and viability >80%, as determined by trypan blue exclusion. After isolation, hepatocytes were suspended in modified medium 199 OR containing 5% fetal calf serum and, where appropriate, plated on collagen-coated 12-well tissue culture plates at a concentration of 0.5×10^6 cells/ml.

For nonparenchymal cells, after *in situ* perfusion of the liver with 0.013 mg% collagenase and 0.25 mg% Pronase, dispersed cells were fractionated on a discontinuous Nycodenz density gradient. The fraction of enriched endothelial and Kupffer cells was subjected to centrifugal elutriation, at flow rates of 18 and 36 ml/min, respectively. After isolation, cells were suspended in modified medium 199 OR containing 20% serum (10% horse/10% calf) for 24 h. Endothelial cells and Kupffer cells were ≥90% pure as assessed by a combination of phase-contrast microscopy, immunocytochemical analysis, and the ability to take up fluorescently labeled acetoacetylated human low-density lipoprotein [20, 21]. Endothelial and Kupffer cells were plated at an initial density of $2.0–5.0 \times 10^6/cm^2$ on collagen-coated and uncoated 24-well tissue culture plates, respectively. Plating efficiency, determined by direct cell counting and trypan blue exclusion, was 75–90%. All cells were maintained in a humidified 2.5% CO_2 incubator at 37°C. In addition, 1 h after LPS plus D-galactosamine injection, hepatic endothelial cells were harvested from fructose-fed (hypertriglyceridemic) rats versus chow-fed controls, and plated on multiwell plates in medium supplemented with inhibitors of nitric oxide synthase at the specified doses for 20 h. The medium was subsequently collected and assayed for nitrite content.

RNA isolation and nitric oxide synthase (NOS) mRNA detection. Total RNA was extracted from endothelial cell pellets using guanidinium isothiocyanate, the concentration of RNA was determined spectrophotometrically, and the integrity of all samples was documented by visualization of 18S and 28S ribosomal bands after electrophoresis through an 0.8% formaldehyde/agarose minigel stained with ethidium bromide.

The cDNA coding for the inducible (NOS2) and the constitutive (NOS3) endothelial cell isoforms of the nitric oxide synthase gene have been previously described [22]. Radiolabeled cRNA was generated by transcription with T7 polymerase using [α-^{32}P]CTP [23]. Specific activity of all radiolabeled transcripts was approximately 0.5×10^9 cpm per microgram.

Total RNA was incubated with $0.5–1.0 \times 10^6$ (Cerenkov) cpm of ^{32}P-labeled cRNA, denatured at 78°C, and hybridized in solution for 16 h at a temperature established as optimal in preliminary experiments (range 55–65°C). Following hybridization, T2 RNase was added to digest unbound label and unprotected mRNA. The protected hybrids were denatured and separated by electrophoresis through a 5% polyacrylamide/urea sequencing gel. Dried gels were applied to X-ray film (Kodak X-O-Mat AR-5) for 12–24 h. Bands corresponding to the protected labeled fragment were normalized to the expression of a normal internal mRNA, S-14 [24].

Nitrite assay. After the isolated liver cells were maintained in culture for approximately 20 h, the medium was removed and immediately assayed for nitrite as a measure of NO [25]. Equal volumes of medium and Greiss reagent (1 part 1% sulfanilamide in 5% phosphoric acid and 0.1% naphthylethylenediamine dihydrochloride in distilled water) were mixed at room temperature. The absorbance of the reaction product was measured at 546 nm and nitrite was cal-

TABLE 1

Plasma Lipid Profiles

	[Triglycerides] (mg/dl)	[Total cholesterol] (mg/dl)
Normolipidemic (chow-fed, control)	98 ± 27	87 ± 16
Hypertriglyceridemic (fructose-fed)	239 ± 53*	114 ± 3*

Note. Values are means ± SD for five rats per group.
* $P < 0.05$.

culated by extrapolation from a standard curve (established by adding known quantities of sodium nitrate to the medium used for cell culture). Raw values were normalized to cell number.

Statistical analysis. Plasma lipid and TNFα and supernatant nitrite (NO) levels were compared using Student's t test, mortality data were compared using χ^2 analysis, and statistical significance was assigned a P value < 0.05.

RESULTS

Animals that consumed a high-fructose diet became hypertriglyceridemic [26], with plasma TG levels that were more than double those of chow-fed, normolipidemic control rats (Table 1). The increased circulating TG was predominantly in the form of TG-rich lipoproteins (VLDL), with a modest increase in the total low-density lipoprotein TG content (data not shown) and total plasma cholesterol concentration of the animals.

To determine whether endogenously produced TG-rich lipoproteins were associated with protection against endotoxicity, rats were injected with a lethal dose of purified LPS plus D-galactosamine and observed for 48 h. Hypertriglyceridemic animals were protected, experiencing a fourfold reduction in mortality when compared with controls (Table 2). In addition, similarly treated animals demonstrated a 30% reduction in peak plasma TNFα concentrations measured 1 h following LPS injection ($P < 0.05$).

TABLE 2

Endotoxin-Induced Mortality and Plasma TNFα Levels

	Mortality (48 h)	[TNFα] (pg/ml)
Normolipidemic (chow-fed, control)	8/12 (67%)	1298 ± 164
Hypertriglyceridemic (fructose-fed)	2/13** (15%)	896 ± 102*

Note. Values for TNFα are means ± SD for six to nine rats per group.
* $P < 0.05$.
** $P < 0.03$.

TABLE 3

Nitric Oxide Production by Specific Liver Cell Types

	nmol nitrite/10^6 cells		
	Hepatocytes	Kupffer cells	Endothelial cells
Normolipidemic (chow-fed, control)	4 ± 3	6 ± 4	3 ± 3
Hypertriglyceridemic (fructose-fed)	3 ± 2	12 ± 8	82 ± 26*

Note. Values are means ± SD for at least four separate experiments.
* $P \leq 0.001$.

We have previously shown that the liver plays an integral role in the catabolism of LPS in a rodent model of sepsis [7, 13, 14]. Thus, hepatic parenchymal and nonparenchymal cells were isolated from hypertriglyceridemic (fructose-fed) rats and normolipidemic (chow-fed) controls, and examined for evidence of LPS-induced cellular activation as measured by NO production [27–30]. There were no significant differences in LPS-induced NO production noted for hepatocytes and hepatic macrophages (Kupffer cells) isolated from hypertriglyceridemic versus control animals. The levels of inducible NO synthase (NOS2) mRNA and NO evident for both cell types were equivalent irrespective of serum lipid concentrations (Table 3). But sinusoidal endothelial cells harvested from the hypertriglyceridemic rats demonstrated increased NOS2 mRNA (Fig. 1) and NO production in response to LPS compared with the normolipidemic controls. The levels of the constitutive endothelial cell isoform (NOS3) were equivalent in the two groups.

The increased NO production by hepatic endothelial cells prompted additional experiments to determine whether NO production was an important component of the fructose-induced protection from endotoxemia.

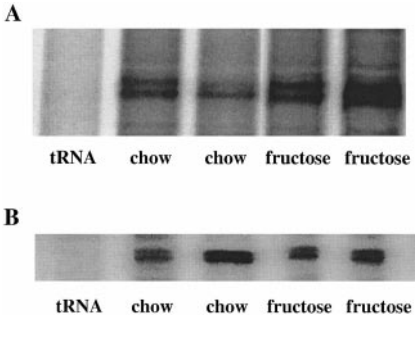

FIG. 1. Representative Northern blot of inducible nitric oxide (NOS2) synthase (A) and constitutive nitric oxide (NOS3) synthase (B) mRNA from rat hepatic endothelial cells. The bands were normalized to the expression of a stable internal mRNA, S-14. Chow, normolipidemic, chow-fed control animal; fructose, hypertriglyceridemic, fructose-fed animal; tRNA: negative control.

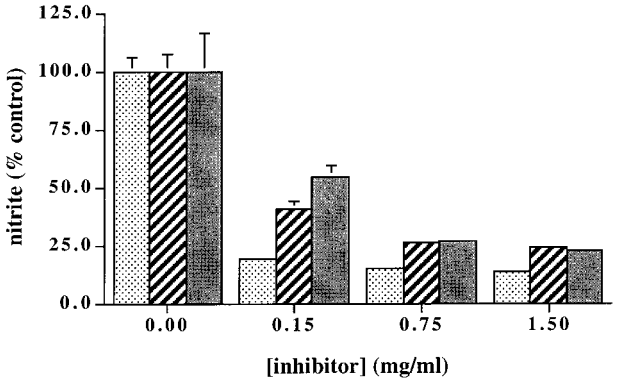

FIG. 2. Effect of selective and nonselective inhibitors of NOS on endothelial cell NO production *in vitro*. Aminoguanidine (stippled bar); N^G-nitro-L-arginine methyl ester (L-NAME, cross-hatched bar); N^G-nitro-L-arginine (L-NNA, closed bar). *$P < 0.01$.

We postulated that if increased NO production is required for the hypertriglyceridemic condition to be protective, then inhibition of NOS activity should render the fructose-fed animals susceptible to LPS-induced mortality. Thus, the effect of both nonselective and selective inhibitors of NOS on LPS-induced mortality and on NO production by hepatic endothelial cells from fructose-fed animals *in vitro* was determined. All three of the inhibitors studied reduced hepatic endothelial cell NO production *in vitro* in a dose-dependent manner (Fig. 2). Over the range of doses used, aminoguanidine appeared a more potent inhibitor of NO production compared with either of the nonselective inhibitors, N^G-nitro-L-arginine methyl ester (L-NAME) or N^G-nitro-L-arginine (L-NNA). When administered *in vivo*, the nonselective inhibitors eliminated the protective effect of the high-fructose diet. The fructose-fed rats injected with either L-NAME or L-NNA experienced increased LPS-induced mortality rates in a dose-dependent manner, such that the protective phenotype could be completely reversed. Curiously, aminoguanidine, considered to be a selective or preferential inhibitor of NOS2 as compared with the other isoforms of the NOS enzyme, did not affect survival in hypertriglyceridemic rats following LPS administration (Table 4). The NOS inhibitors had no effect on mortality in chow-fed control animals and were not directly toxic to untreated animals.

DISCUSSION

Triglyceride-rich lipoproteins have been shown to protect against endotoxicity in various rodent models of sepsis [7–9, 12]. Through formation of lipoprotein–LPS complexes the lipopolysaccharide macromolecule is principally removed from the circulation by the liver, resulting in reduced cytokine production and LPS-induced mortality [7, 11, 13]. Here we demonstrate for the first time that elevated levels of endogenous TG-rich lipoproteins may result in protection against LPS. After 2 weeks of consuming a fructose-enriched diet rats developed a stable, hypertriglyceridemic condition which was associated with markedly reduced LPS-induced TNFα production and mortality.

Recognizing the integral role of the liver in the catabolism of LPS, we subsequently examined the impact of injected lipopolysaccharide on specific liver cell NO production as an indicator of LPS-induced cellular activation. There were no differences in NO production by either cultured hepatocytes or Kupffer cells isolated from hypertriglyceridemic versus control rats in response to LPS. However, there was a noted increase in NOS2 mRNA levels and NO production by hepatic endothelial cells harvested from the hypertriglyceridemic rats versus the normolipidemic, chow-fed controls. The increased production of NO by endothelial cells was observed only in the fructose-fed rats following LPS administration, as cells isolated from untreated hypertriglyceridemic animals yielded low, basal levels of NO (Table 3).

To further understand the impact of the fructose diet on endothelial cell NO production, specifically which isoform(s) of the NOS enzyme was involved, we examined the effect of NOS inhibitors both on NO production *in vitro* and on LPS-induced lethality. One might reasonably postulate, given the large amount of NO produced by the hepatic endothelial cells from hypertriglyceridemic (fructose-fed) rats, that the inducible NOS2 isoform would be responsible for this markedly increased activity. Indeed, the levels of NOS2 mRNA were increased in the endothelial cells from the fructose-fed rats following LPS as compared with endothelial cells from chow-fed controls. And aminoguanidine, a selective inhibitor of NOS2, exerted a potent

TABLE 4

Effect of Nitric Oxide Synthase Inhibition on LPS-Induced Mortality

[Inhibitor] (mg/ml)	Mortality (48 h)		
	Aminoguanidine	L-NAME	L-NNA
0	8/33 (24%)	8/33 (24%)	8/33 (24%)
10	0/9 (0%)	5/9 (56%)	n.d.
50	2/9 (22%)	9/9* (100%)	n.d.
100	2/11 (18%)	9/10** (90%)	5/5** (100%)

Note. L-NAME, N^G-nitro-L-arginine methyl ester; L-NNA, N^G-nitro-L-arginine.

* $P < 0.005$.

** $P < 0.04$.

inhibitory effect on endothelial cell NO production *in vitro*. Yet, administration of aminoguanidine *in vivo* had no effect on LPS-induced mortality in hypertriglyceridemic rats, while the nonselective inhibitors inhibited endothelial cell NO production *in vitro* and completely abolished the fructose-induced protection against endotoxemia *in vivo*. Two possible explanations for why aminoguanidine did not affect rodent mortality are that the dose administered was inadequate to inhibit NOS2 and that the inhibitor may have been pharmacologically unavailable to the hepatic endothelial cells. But both potential explanations are unlikely since the range of dosages used in this study has been shown by other investigators to effectively inhibit NOS2 both *in vitro* [31, 32] and *in vivo* [33–38]. Also, we observed excellent *in vitro* inhibition of endothelial cell NO production at the lowest dosage used.

The inability of aminoguanidine versus the nonselective inhibitors to affect LPS-induced mortality is in agreement with data from other investigators who have also examined the impact of NOS inhibitors on the response of rodents to LPS *in vivo* [34, 37]. Tracey *et al*. injected rodents with LPS plus either selective or nonselective NOS inhibitors [34]. As in our study, animals injected with the nonselective NOS inhibitors L-NAME and L-NNA yielded an increased mortality rate as compared with rodents in which NOS2 was specifically inhibited by aminoguanidine or dexamethasone administration. As these authors observed, and as supported by our *in vitro* data (Fig. 2), the inhibition of NOS2 resulted in decreased overall NO production without effect on LPS-induced mortality. Also, while examining the effect of aminoguanidine in a rat model of acute endotoxemia, Hock *et al*. showed that even though inhibition of NOS2 resulted in decreased systemic NO production, many of the deleterious cardiovascular effects of LPS were unaffected [37]. These data likely indicate that while NOS2 is responsible for the large increases in systemic (circulating) NO observed in these rodents after LPS, it is the constitutive isoforms of the enzyme (NOS1/NOS3) that are necessary for the animal to survive an endotoxic challenge [33]. While aminoguanidine is effective at inhibiting the NOS2-dependent, systemic overproduction of NO, the organ failure and lethality induced by LPS in these rodent models of sepsis are mediated by the constitutive isoform (NOS3). Further, these data suggest that the relatively low levels of NO produced within cells by the constitutive isoforms of NOS are apparently critical for protection against LPS-induced organ failure and death. While there are data that inhibition of NOS2 by aminoguanidine can successfully attenuate LPS-induced organ failure in rats [35, 39], these findings have not been widely duplicated.

The overall significance of increased hepatic endothelial cell NO production in response to LPS is a complex matter. Other investigators have reported data supporting a role for endothelial cell-derived NO as an important regulator of vascular tone and, thus, blood flow within the liver, suggesting that increased NO production could prevent hepatic ischemia during sepsis and thus protect against ischemia–reperfusion injury [40–42]. More recently there have been reports regarding a cytoprotective role for NO within the liver during acute inflammation and cytokine-mediated cellular injury. NO may protect hepatocytes from TNFα-induced toxicity and apoptosis, possibly through the induction of heat shock protein 70 [43]. Whereas clarifying the precise role of NO within the liver during acute inflammatory states is beyond the scope of this study, our findings add to the data in support of NO as a critical mediator of the hepatic response to infection and other proinflammatory stimuli [44]. Interestingly, the exact mechanism by which fructose-induced hypertriglyceridemia promotes increased NO production by hepatic endothelial cells in response to LPS and the mechanism by which the specific isoforms are affected are not known. Perhaps TG-rich lipoproteins can modulate the response of hepatic nonparenchymal cells to LPS, as has been shown for hepatocytes [13].

The high-fructose diet administered in this study can result in mild hypertension and hyperinsulinemia, in addition to hypertriglyceridemia. Thus, it is quite possible that the protective phenotype observed results from these other effects and not the hyperlipoproteinemia or some additional physiological alteration that has yet to be described. However, there are ample data supporting the capacity of lipoproteins to neutralize circulating LPS and no studies to date correlating hypertension or hyperinsulinemia with such protection.

SUMMARY

We have demonstrated that endogenous TG-rich lipoproteins can protect against endotoxicity *in vivo* through a NO-dependent mechanism. Since the effective neutralization of the injected LPS by circulating hepatic lipoproteins occurred completely *in vivo*, these findings lend support to the possible therapeutic application of a lipid-based therapy for the treatment of Gram-negative sepsis.

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