Chylomicrons promote intestinal absorption of lipopolysaccharides

Sarbani Ghoshal,* Jassir Witta,* Jian Zhong,* Willem de Villiers,*,†,§ and Erik Eckhardt[1],*,†,§

Department of Internal Medicine, Division of Digestive Diseases and Nutrition,* Graduate Center for Nutritional Sciences,† and Cardiovascular Research Center,§ University of Kentucky, Lexington, KY

Abstract Recent data suggest that dietary fat promotes intestinal absorption of lipopolysaccharides (LPS) from the gut microflora, which might contribute to various inflammatory disorders. The mechanism of fat-induced LPS absorption is unclear, however. Intestinal-epithelial cells can internalize LPS from the apical surface and transport LPS to the Golgi. The Golgi complex also contains newly formed chylomicrons, the lipoproteins that transport dietary long-chain fat through mesenteric lymph and blood. Because LPS has affinity for chylomicrons, we hypothesized that chylomicron formation promotes LPS absorption. In agreement with our hypothesis, we found that CaCo-2 cells released more cell-associated LPS after incubation with oleic-acid (OA), a long-chain fatty acid that induces chylomicron formation, than with butyric acid (BA), a short-chain fatty acid that does not induce chylomicron formation. Moreover, the effect of OA was blocked by the inhibitor of chylomicron formation, Pluronic L-81. We also observed that intragastric triolein (TO) gavage was followed by increased plasma LPS, whereas gavage with tributyrin (TB), or TO plus Pluronic L-81, was not. Most intestinally absorbed LPS was present on chylomicron remnants (CM-R) in the blood. Chylomicron formation also promoted transport of LPS through mesenteric lymph nodes (MLN) and the production of TNFα mRNA in the MLN.█ Together, our data suggest that intestinal epithelial cells may release LPS on chylomicrons from cell-associated pools. Chylomicron-associated LPS may contribute to postprandial inflammatory responses or chronic diet-induced inflammation in chylomicron target tissues.— Ghoshal, S., J. Witta, J. Zhong, W. de Villiers, and E. Eckhardt. **Chylomicrons promote intestinal absorption of lipopolysaccharides.** *J. Lipid Res.* 2009. **50:** 90–97.

A high-fat diet increases the risk for acute and chronic inflammation (1, 2) and for chronic inflammatory diseases such as atherosclerosis and diabetes (3–5). The proinflammatory effect of fatty diets has mainly been attributed to the inflammatory properties of dietary fatty acids. Indeed, fatty acids are known to be able to modulate the immune system (6) and to activate the innate immune receptor TLR4, which might contribute to inflammation of adipose tissue in obesity (7, 8). Some dietary fatty acids are also susceptible to oxidation before and after their intestinal absorption, which may promote inflammatory responses relevant to cardiovascular (9) and other diseases (10). However, excess dietary fat not only increases systemic exposure to potentially proinflammatory free fatty acids and their derivatives, but its intestinal absorption was recently found to also facilitate the absorption of highly proinflammatory bacterial lipopolysaccharides (LPS) from the gut (11, 12). This is highly interesting, because the absorbed LPS might affect whole body inflammation and interfere with metabolism and the function of the immune system.

Dietary long-chain fat is transported from the absorptive enterocyte to extra-intestinal tissue after its incorporation as triglycerides into chylomicrons and its release into mesenteric lymph. Once in the systemic circulation, these large lipoprotein particles can release significant amounts of free fatty acids upon interaction with lipoprotein lipases tethered to the endothelium, and this might partially explain the putative proinflammatory and proatherogenic potential of these lipoproteins (1, 13, 14). However, chylomicrons also have high affinity for LPS (15–17) and thus not only transport postprandial fat, but likely also significant amounts of concomitantly absorbed gut LPS. Whereas sequestration of absorbed LPS on chylomicrons would reduce LPS toxicity and enhance its hepatic clearance (17), it nevertheless is possible that the inflammatory effect of chylomicrons could correlate with their LPS content. Moreover, excess chylomicron formation during high-fat dieting, which would lead to prolonged chylomicronemia, could augment extra-hepatic LPS exposure, perhaps increasing the risk for atherosclerosis and other metabolic or inflammatory disorders.

Abbreviations: apoB, apolipoprotein B; BA, butyric acid; CM-R, chylomicron remnants; LCT, long-chain triglycerides; LPS, lipopolysaccharides; MCT, medium chain triglycerides; MLN, mesenteric lymph nodes; OA, oleic acid; TB, tributyrin; TO, triolein.
[1] To whom correspondence should be addressed.
    e-mail: eeckh2@uky.edu

An important question therefore is how dietary fat promotes intestinal LPS absorption. One possibility is that dietary fat promotes paracellular leakage of LPS across the intestinal epithelium. This is supported by the recent observations that intestinal-epithelial tight-junction integrity is compromised in obese mice (18) and by studies demonstrating that experimental intestinal luminal exposure to oleic acid (OA) can cause small-intestinal epithelial damage (19, 20). An alternative possibility could be that LPS enters the body proper by transcellular transport through intestinal epithelial cells. This could occur through so called intestinal-epithelial microfold cells (M-cells), which are permeable to bacteria and macromolecules and facilitate sampling of gut antigens by the underlying lymphoid tissue (21).

Uptake of bacteria and LPS may not be limited to M-cells, however, and has also been demonstrated to occur in absorptive enterocytes (22) where it may relate to the known induction of LPS tolerance in these cells (23, 24). Interestingly, LPS which is internalized by intestinal epithelial cells is transported to the Golgi compartment of the enterocyte (23), which is also where newly assembled chylomicrons are located prior to their basolateral secretion (25). Therefore, we hypothesized that chylomicron secretion would result in concomitant basolateral secretion of LPS from cell-associated pools.

In the present study, we explored the mechanism of intestinal LPS absorption. We observed that cultured CaCo-2 cells basolaterally secrete cell-associated LPS during OA-induced chylomicron formation, which was not observed when the cells were incubated with short-chain butyric acid (BA), which does not induce chylomicron formation or when OA was added together with the inhibitor of chylomicron formation, Pluronic L-81 (Pl-81). Using in vivo studies, we observed that dietary triolein (TO) significantly promoted LPS absorption into blood and mesenteric lymph nodes (MLN) compared with tributyrin (TB) and caused significant increases of TNFα mRNA. TO-dependent LPS absorption was completely blocked by Pl-81, even though this inhibitor does not interfere with fat uptake into the enterocytes. Together, these results strongly suggest that chylomicron formation promotes LPS absorption from the gut and suggest that the proinflammatory effect of chylomicrons may in part relate to its enrichment with gut LPS.

MATERIALS AND METHODS

Cells, media, reagents

CaCo-2 cells, CMT93 cells and T84 cells were purchased from ATCC (Manassas, VA). CaCo-2 cells were grown in HEPES- and bicarbonate-buffered DMEM/Ham's F12 medium supplemented with 5% fetal calf serum (FCS) and antibiotics. The cells were subcultured once a week for up to 30 passages (final passage number ~50). For experiments, cells from a near-confluent 75 cm^2 flask were trypsinized and resuspended in 10 ml medium; 0.1 ml of this suspension was seeded per cm^2 (26) of permeable supports (collagen coated "transwells," 12 or 6 wells/plate; pore size 3 μm, Corning Corp.) and were grown for 21 days (26) with triweekly medium changes. CMT93 cells were grown in DMEM with 10%

fetal calf serum and antibiotics and were used within 10 passages. CMT93 cells were seeded on similar inserts as the CaCo-2 cells and at similar densities but were used within 1 day of confluency, after which the transepithelial potential difference exceeded 400Ω/cm^2. T84 cells were grown and seeded on membrane supports as described for CaCo-2 cells. MEM-based enriched medium requiring little or no serum ("Opti-MEM") was purchased from Invitrogen, and FCS from Hyclone. Sodium-salts of taurocholic-, butyric- (BA) and oleic- (OA) acids, TO, and TB were from Sigma Aldrich, whereas medium chain triglycerides (MCT) oil (consisting of triglycerides containing mainly octanoic and decanoic acids) was from Novartis. Pluronic L-81 was a generous gift from BASF corporation (Florham Park, NJ) and was used at 3% (by volume) in the gavage oil [comparable to amounts used to block chylomicron formation in gavaged rats (27)] and at 0.2% in the in vitro experiments (26). LPS (E. coli 0114:B4) was purchased from Sigma Aldrich, and the Limulus Amoebocyte Lysate assay from Lonza Biosciences. [^{3}H] Labeled high molecular weight dextran and ^{125}NaI were obtained from Amersham, [^{3}H]retinol from American Radiolabeled Chemicals (St. Louis, MO). All other reagents were from Sigma Aldrich.

Animals

Male C57Bl/6 mice were purchased from the Jackson Laboratory and were allowed to acclimatize for 2 weeks prior to the experiments. LDL-receptor knockout mice on a C57Bl/6 background were a gift from Dr. Alan Daugherty. The age of the mice at the onset of the experiments did not exceed 11 weeks. A light/dark cycle of 12/12 h was maintained and the mice had adlib access to water and food. All animal experiments had institutional approval.

Radiolabeling of LPS

The ^{125}I-labeled LPS was kindly donated by the van der Westhuyzen laboratory. The LPS was labeled according to the chloramine-T protocol (28, 29). Briefly, 1 mg of LPS was dissolved in 0.05 M borate buffer (pH8) and incubated with 50 mM p-OH methylbenzimidate at 37°C overnight. After extensive dialysis and incubation of the treated LPS with Na^{125}I, unbound radiolabel was removed by overnight dialysis against sterile saline in the cold.

Immunoblotting

Fifteen μl of plasma or basolateral media from transwell cultures were diluted with 4× SDS sample buffer. The samples were not boiled but reduced with β-mercapto-ethanol and were subjected to SDS-PAGE on a 4–20% Tris-Glycine gel before being transferred onto a PVDF membrane. Apolipoprotein B (apoB)-48 was identified with a polyclonal anti-human ApoB antibody (Calbiochem; cross-reacting with mouse ApoB) as a band migrating at around 250 kDa.

Isolation of CM-R

Plasma (0.175 ml) of LDL receptor knockout mice was centrifuged in a Beckman Airfuge (15 min at 110,000 RPM) in order to float chylomicron-remnant particles chylomicron remnants (CM-R) (30). The top layer (<10% by volume) was carefully isolated and resuspended in endotoxin-free water to the initial volume.

Detection of plasma LPS

Undiluted plasma samples were directly subjected to the Limulus assay as per the manufacturer's instructions. All pipette tips and tubes were certified endotoxin free.

RT-PCR

RNA was transcribed into cDNA with MMLV reverse transcriptase as part of the Biochain Optimax kit, using random hexamers. Expression of TNFα cDNA was quantified with real-time PCR using the primer pairs 5′-AGC-CGA-TGG-GTT-GTA-CCT / 5′-TGA-GTT-GGT-CCC-CCT-TCT (TNFα) and 5′-CGG-CTA-CCA-CAT-CCA-AGG-AA / 5′-GCT-GGA-ATT-ACC-GCG-GCT (18S rRNA) with an annealing temperature of 60°C.

Statistical analyses

All data are represented as average ± SD. Groups were compared by ANOVA and Bonferroni's posthoc tests or by Student's *t*-tests. Differences between groups were considered statistically significant when $P < 0.05$.

RESULTS

Ingestion of LCT causes an increase in plasma LPS, which is mainly associated with the chylomicron (remnant) fraction

To test whether dietary long-chain triglycerides (LCT) promote absorption of endogenous LPS into the blood, we measured bioactive LPS in the plasma of mice gavaged with TO or TB. We used LDL-receptor mice since these had previously been shown to have delayed CM-R clearance (31), allowing us to isolate more CM-R than would have been possible with wild-type mice. The mice were fasted (4 h) and then gavaged with 0.2 ml TO or TB. Ninety minutes later, the animals were euthanatized, blood was collected, and 0.175 ml of plasma was centrifuged in a Beckman Airfuge (15 min at 110,000 RPM) in order to float CM-R (30). Bioactive LPS in total plasma and in CM-R fractions resuspended in endotoxin-free water to the same volume as the initially centrifuged sample was measured with a Limulus Amoebocyte Lysate assay (32). As expected, significantly more fat was floated from the plasma of TO-gavaged mice (not shown), and their plasma contained more apoB-48 (**Fig. 1B**). Moreover, the plasma of TO-gavaged mice contained significantly more bioactive LPS, which was mainly associated with the CM-R fraction (Fig. 1A). We conclude that long-chain dietary fatty acids promote absorption of endogenous, bioactive LPS and that most absorbed LPS is associated with CM-R particles.

Inhibition of chylomicron formation blocks long-chain triglyceride-mediated intestinal absorption of LPS

To further test whether chylomicrons are involved in the absorption of bacterial material from the intestine into the blood, fasted (4 h) wild-type mice received ^{125}I-labeled LPS by oral gavage and were subsequently fasted for 6 h. Thereafter, the mice received a second gavage with 0.1 ml of either TO, TB, or TO plus 3% (by volume) of the inhibitor of chylomicron formation, Pluronic L-81 (Pl-81) (27, 33, 34). One half h after the second gavage, the mice were sacrificed and blood was collected and analyzed for radioactivity. As shown in **Fig. 2**, TO-gavaged mice had significantly higher amounts of radioactivity in the blood than mice gavaged with TB. Moreover, Pl-81 blocked the effect of TO. Thus, long-chain dietary fat stimulates intestinal LPS ab-
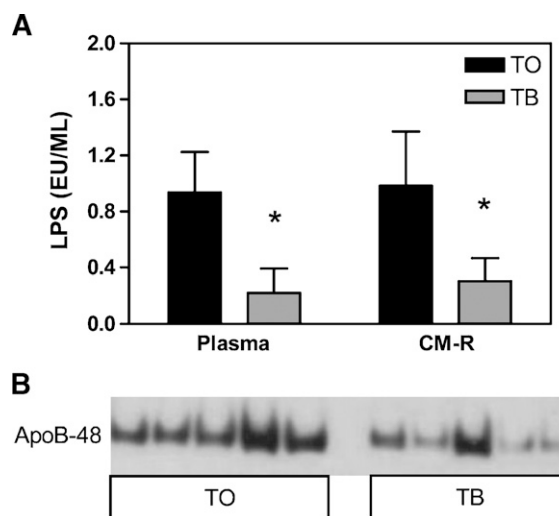

Fig. 1. Dietary long-chain triglycerides (LCT) increase plasma lipopolysaccharides (LPS), which is mainly associated with chylomicron-remnants. A: LDL receptor $^{-/-}$ mice were fasted for 4 h and then received an intragastric bolus of triolein (TO) or tributyrin (TB). Ninety minutes later, plasma was centrifuged in a micro-ultracentrifuge ("Airfuge") to float chylomicron remnants (CM-R) (30), and the CM-R were resuspended in endotoxin-free water to the same volume as before centrifugation. Panel B shows apolipoprotein B (apoB)-48 immunoreactivity in 15 µl plasma. N = 5 per group, shown are averages ± SD. The asterisk denotes statistical significance at $P < 0.05$ (*t*-test). The experiment was carried out twice with similar results.

sorption into the blood in a mechanism dependent on chylomicron formation.

Long-chain fatty acids promote absorption of LPS into MLN in a chylomicron-dependent manner

Chylomicrons and their remnants are transported through mesenteric lymph before entering the blood stream and being cleared in various tissues. To determine whether
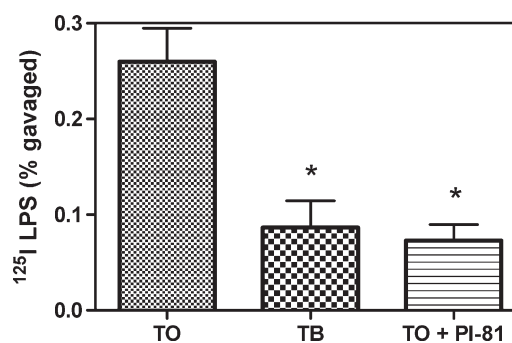

Fig. 2. Chylomicron formation promotes intestinal absorption of ^{125}I-LPS into blood. Mice were fasted 4 h and were subsequently gavaged with 0.1 ml phosphate-buffered saline containing ^{125}I-LPS. After another 6 h fast, mice received a second gavage with 0.1 ml of TO, TB, or TO plus Pl-81. Thirty minutes later, the mice were euthanatized and radioactivity was measured in 0.1 ml blood. Fractional absorption was estimated assuming a total blood volume of 1 ml per mouse. N = 4 per group, shown are average ± SD. The asterisk denotes statistical significance at $P < 0.01$ compared with TO (ANOVA).

chylomicron-dependent fat absorption correlated with transport of LPS through mesenteric lymph, fasted wild-type mice were gavaged with ^{125}I-LPS and subsequently with LCT (TO) plus or minus Pl-81. One h later, MLN were isolated and analyzed for radioactivity after homogenization. Consistent with our hypothesis that chylomicron formation enhances absorption of LPS, we observed that Pl-81 significantly decreased the amount of radioactivity in the MLN after LCT gavage (**Fig. 3A**). In separate experiments, a radiolabeled marker for chylomicron formation and transport, retinol (34, 35), was gavaged instead of LPS in order to determine whether the LCT gavage had indeed resulted in increased chylomicron transport through the MLN. As shown in Fig. 3B, LCT gavage significantly increased MLN retinol content compared with gavage of LCT plus Pl-81. Together, these observations strongly suggest that absorbed bacterial material is transported through mesenteric lymph consistent with an absorption mechanism that depends on chylomicron formation.

The finding that intestinal bacterial material is deposited in chylomicron target tissue suggests that chylomicrons might cause inflammatory responses in such tissue, at least in part by enriching the tissue with gut-derived bacterial material. To investigate this possibility, fasted mice were gavaged with 1 μg LPS together with LCT, MCT, or LCT plus Pluronic L-81, and the MLN were obtained 90 min later. TNFα mRNA was measured by RT-PCR. As shown in
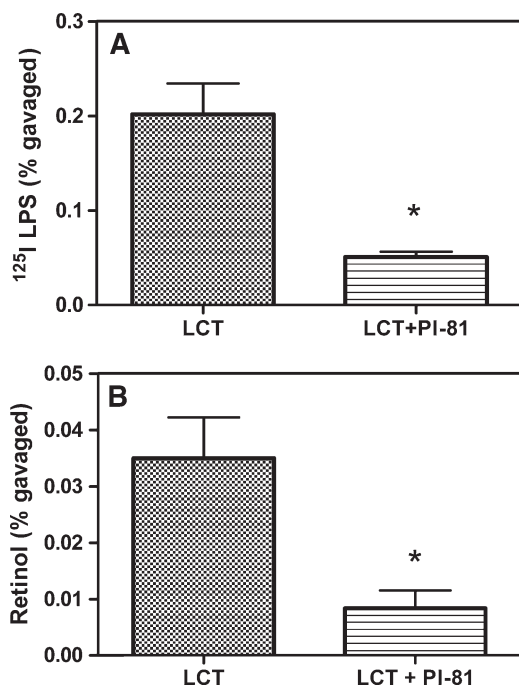
Fig. 4, LCT gavage significantly increased TNFα mRNA levels in MLN as compared with MCT or LCT in the presence of the chylomicron inhibitor Pl-81. Although it cannot be excluded that fatty acids might have contributed to this effect (see Discussion), the data in Fig. 3 suggest that chylomicron-dependent absorption of LPS should be considered as a novel contributor to inflammatory responses.

Chylomicron formation enhances basolateral secretion of cell-associated LPS by CaCo-2 cells

To further investigate the mechanism through which dietary long-chain fat enhances LPS absorption, we used CaCo-2 cells, cultured on permeable supports ("transwells") as a model for small-intestinal enterocytes (36). The cells were incubated overnight with ^{125}I-labeled or unlabeled (0.1 mg/ml) LPS from the apical side, and unbound LPS was removed from the cells by extensive washing from both sides. We did not detect any ^{125}I LPS in subsequent washes of apical and basolateral compartments (not shown), in line with a previous study in which identical wash procedures were shown to completely remove unbound LPS (37). Thereafter, the cells were incubated from the apical side with Opti-mem containing 0.5 mM taurocholate and either 1.6 mM of OA to induce chylomicron formation (26) or BA as control. Twelve h later, the basolateral medium was collected and analyzed for LPS (Limulus Amoebocyte Lysate assay or radiolabel), and chylomicron formation was assessed by Western blot analysis of apoB-48 in the basolateral milieu. As shown in **Fig. 5A**, OA induced more pronounced apoB48 release into the basolateral medium than BA or OA plus Pl-81. Moreover, when unlabeled LPS was added, OA significantly stimulated basolateral secre-
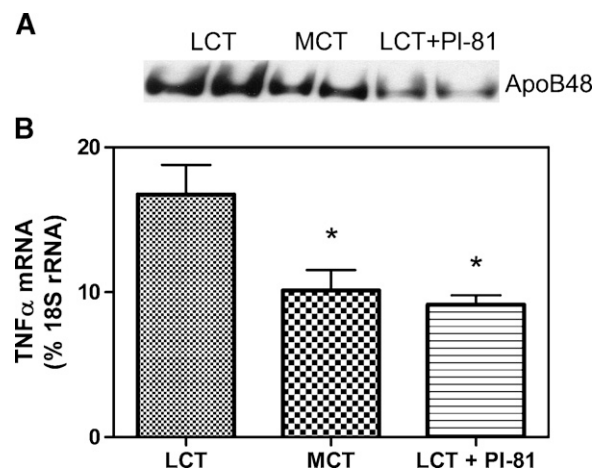

Fig. 3. Chylomicron formation increases deposition of intestinal LPS in mesenteric lymph nodes (MLN). Fasted mice were gavaged with 0.1 ml of ^{125}I-LPS (A) or [3H]retinol (B) and, after a 6 h fast, with 0.1 ml of long-chain triglyceride (TO) or LCT plus 3% Pl-81. Ninety minutes later, the mice were euthanized and radioactivity was measured in homogenates of combined mesenteric lymph nodes (MLN) per animal. Shown are the average of n = 3 ± SD. Asterisk denotes statistically significant difference with TO at $P <$ 0.01 (t-test).

Fig. 4. Dietary LCT augment TNFα expression in mesenteric lymph nodes (MLN). Fasted mice were gavaged with 1 μg LPS together with 0.2 ml of TO (LCT), medium chain triglycerides (MCT) oil, or LCT plus Pluronic L-81 (3% by volume). Ninety minutes later, RNA was isolated from pooled MLN of each mouse and TNFα mRNA, relative to 18S rRNA, was measured by quantitative RT-PCR (B). Shown is the average of five animals per group; error bars indicate SD. The asterisk denotes statistically significant difference ($P <$ 0.05; ANOVA). Panel A shows corresponding ApoB-48 levels in the plasma of the mice.
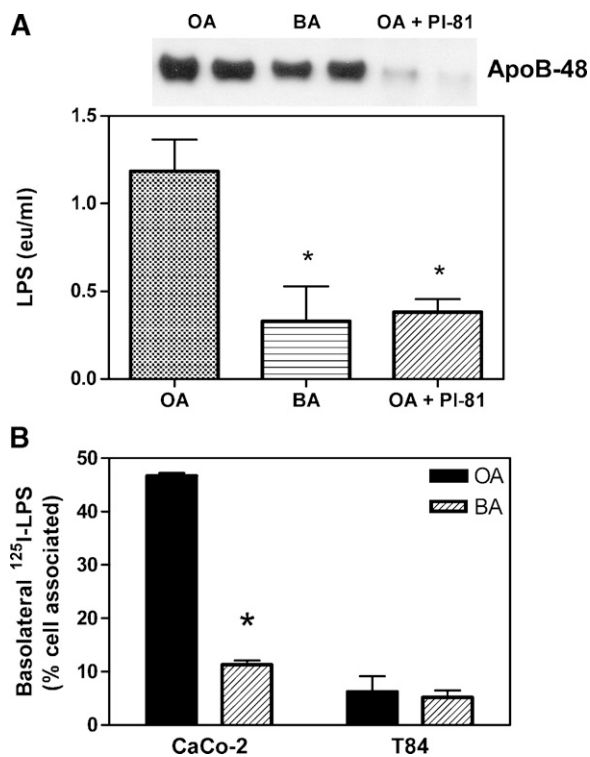
Fig. 5. Chylomicron formation enhances basolateral secretion of cell-associated LPS. CaCo-2 or T84 cells, grown on transwells, were incubated overnight with 0.1 mg/ml unlabeled LPS (A) or ^{125}I-LPS (B) in the apical chamber. After extensive washing to remove unbound LPS (37), cells were incubated from the apical surface with Opti-mem containing 0.5 mM taurocholate and 1.6 mM of either OA [to induce chylomicron formation (26)] or BA (control), and the basolateral milieu was analyzed 12 h later by a LAL assay (A) or in a γ counter (B). The blot in A shows apoB-48 immunoreactivity in the basolateral milieu of CaCo-2 cells after the fatty acid treatment. All results are represented as average ± SD (n = 4); the asterisk denotes statistically significant differences between BA and OA treatments [$P < 0.05$, ANOVA (A) and t-test (B)].

tion of bioactive LPS (Fig. 5A) compared with BA. Because no LPS was present in the apical compartment during OA treatment, all LPS was apparently derived from cell-associated pools. Experiments in which the added LPS was labeled with ^{125}I to allow for measurement of cell-associated LPS showed similar results (Fig. 5B). Moreover, T84 cells, a colonic epithelial cell line that did not release apoB into the basolateral medium (not shown), did not secrete ^{125}I-LPS after addition of OA (Fig. 5B). Although the above results show that LPS is secreted from cell-associated pools, we nevertheless also tested whether OA could impair epithelial tight junction integrity. Therefore, we grew CMT93 cells on transwells until tight junctions were formed and incubated the cells from the apical side with 1.6 mM OA or BA together with 0.5 mM taurocholate and [^{3}H]labeled high-molecular weight dextran, and samples of the basolateral medium were counted at various times. As shown in **Fig. 6,** there was no significant difference in transepithelial dextran transport between the various treatment groups. Together, these results are consistent with a model in which chylomicron formation is associated with basolateral secretion of cell-associated LPS.
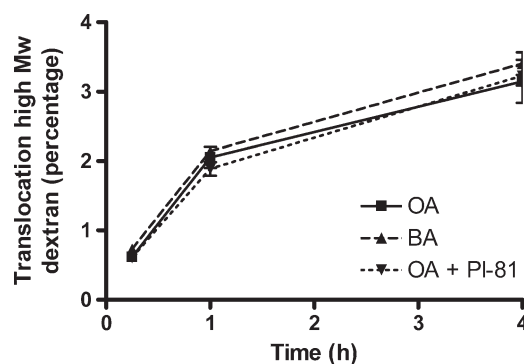
Fig. 6. Oleic acid (OA) does not affect tight-junction function in cultured intestinal epithelial cells. CMT93 cells were grown on transwell inserts until the transepithelial potential difference had reached >400Ω/cm^2. Cells were then incubated on the apical side with Opti-MEM containing 1% serum and taurocholate (0.5 mM) supplemented with OA, BA (1.6 mM each), or OA+PI-81 (2 μg/ml). All apical compartments also contained 1μCi [^{3}H]Dextran (70 kDa). Basolateral samples were taken at indicated time points and the percentage translocation was calculated. Shown are averages of three points, with SD. The experiment was repeated twice with similar results.

DISCUSSION

Our intestines are colonized by large amounts of microorganisms that significantly outnumber our own cells (38). As a consequence, more than 1 g of LPS can be found in the gut lumen (38). Even small amounts of this highly proinflammatory substance could elicit strong inflammatory responses in the body proper, and it is therefore thought that the gut epithelium acts to effectively block the "translocation" of LPS and other microbial proinflammatory substances. However, it was shown several decades ago that small amounts of LPS are absorbed from the gut in healthy animals (39). In that study it was shown that intragastrically applied ^{125}I-LPS, similarly prepared as the LPS in our present study, could be extracted in its entirety from the liver, thus showing that bioactive LPS is being absorbed from the gut and circulated through the body. Moreover, bioactive LPS is detectable in low amounts in the blood of healthy human subjects even in the apparent absence of infections (40, 41), which would suggest that small amounts of LPS are being continuously absorbed from the gut, perhaps as part of a normal physiological process of yet unknown relevance.

Excessive LPS absorption, however, could evidently be harmful and could lead to acute or chronic inflammation. Increased LPS absorption, for example, could exacerbate the risk for several chronic diseases, such as alcoholic liver injury (42), nonalcoholic steatohepatitis (18), HIV/AIDS (40), and inflammatory bowel disease (43, 44). Therefore, it is important to know the mechanism of LPS absorption from the gut, and recent work suggests that the diet may play an important role since LPS absorption from the gut was found to be associated with the ingestion of dietary fat (11, 12). This is highly interesting, because excessive fat intake is a risk factor for the development of a variety of inflammatory diseases, including diabetes and atherosclerosis (3–5, 45). Traditionally, this increased risk has been

associated with biological effects of dietary fat, such as a direct activation by free fatty acids of innate immune signaling pathways (7, 8) or by increasing oxidized fatty acids (as reviewed in Refs.9, 10). However, the recent observation that dietary fat promotes intestinal LPS absorption suggests that gut LPS should be considered as a potentially contributing factor to diseases that are linked to excessive fat intake.

The recent reports on the association between intestinal absorption of fat and of LPS have not provided a mechanism as to how dietary fat increases intestinal absorption of LPS, and our study was aimed at shedding light onto this issue. In theory, dietary fat could increase LPS absorption in several ways. One way would be through promotion of paracellular uptake of macromolecules as a result of deleterious effects of fatty acids on tight-junction integrity (19). Our data do not support such a mechanism, for two reasons. First, our in vivo experiments showed that Pl-81, a specific inhibitor of chylomicron formation (46, 47), completely blocked TO-dependent intestinal absorption of LPS. Because Pl-81 does not affect the uptake of lipid into the enterocyte (46, 47), a process which requires triglyceride hydrolysis into fatty acids and monoacyl-glycerols, it is unlikely that Pl-81 caused a decrease in luminal free OA content. Therefore, the intestinal epithelium of the mice that had received TO plus PL-81 likely had to endure the same OA-related epithelial stress as the mice that had received TO only. Second, in our in vivo experiment in Fig. 2, we waited 6 h between the LPS gavage and the fat gavage, arguing that luminal free ^{125}I-LPS content would be strongly reduced in the duodenum and jejunum at the time of the fat gavage. Third, our in vitro data show that basolateral secretion of LPS upon addition of OA occurred in absence of LPS in the apical milieu, which suggests that the secreted LPS was derived from cell-associated pools. Fourth, our data show that OA does not cause tight junction disruption in cultured intestinal-epithelial cells. Even if small amounts of LPS had remained in the apical chamber, the concentration (1.6 mM) of OA used in our and others' CaCo-2 experiments (26) was almost 10-fold lower than the concentration required to induce tight junction leakage in a recent study with CaCo-2 cells (48) and about 20 times lower than concentrations used to disrupt intestinal epithelial tight junctions in vivo (19). An alternative mechanism explaining fatty-acid dependent LPS absorption involves internalization of LPS by the enterocyte, followed by association of some of the internalized LPS with chylomicrons and concomitant basolateral secretion of LPS with the chylomicrons or by association of independently transcytosed LPS with newly released chylomicrons. Intestinal epithelial cells have indeed been shown to internalize LPS (23) and to express LPS receptors such as MD2/TLR4 (22, 24, 49, 50), although CD14 expression is low (51). Moreover, the intestinal epithelium is known to express scavenger receptors such as SR-BI (52–55), which is known to mediate binding and internalization of LPS in other cell types (56). We have indeed found evidence for involvement of SR-BI in the uptake of LPS into the intestinal epithelium (Eckhardt et al., unpublished observations). Although some LPS acquired by the intestinal epithelial cell remains associated with the cell membrane (37), significant amounts of LPS are internalized and directed to the Golgi, where most TLR4 resides (23). Because the Golgi also is a major compartment in chylomicron transport to the basolateral membrane (25, 57, 58), and because LPS has strong affinity for chylomicrons (15), it is not implausible to assume that LPS might associate with chylomicrons within the enterocyte. This possibility is further supported by the fact that intestinal epithelial cells express LPS binding protein (59), which mediates the association of LPS with chylomicron particles in the circulation (15). Thus, our data that suggest that LPS is secreted from cell-associated pools in a chylomicron-dependent manner could be partially explained by loading of cell-associated LPS onto nascent chylomicrons.

In the present study, we also show for the first time that dietary LCT stimulate TNFα expression in the MLN (Fig. 4), an important center of intestinal immunity (60). It is possible that OA could have contributed to this proinflammatory effect, because high doses of fatty acids have been shown to activate TLR4 (7, 8). However, chylomicron-associated triglycerides are not subject to significant lipoprotein-lipase dependent hydrolysis in the lymphatics (61) but are mainly hydrolyzed by lipases associated with the endothelium of blood vessels (62). Thus, it is unlikely that significant amounts of fatty acids had been released by newly formed chylomicrons during the early phases of their transport through the mesenteric lymph. Our data also show for the first time that chylomicron formation correlates with increased deposition of LPS in the MLN (Fig. 3). This novel observation could be of relevance to inflammatory bowel disease, since long-chain dietary fat is considered to be harmful to a significant subpopulation of patients suffering from Crohn's Disease (63). Our results would suggest that blocking chylomicron formation, by dietary or pharmacological means, could perhaps be of benefit in Crohn's Disease by preventing activation of immune cells in the MLN by LPS.

Together, our data support a novel mechanism by which dietary fat may affect inflammatory responses in the body. Our findings support a model in which intestinal epithelial cells, which acquire LPS and perhaps other bacterial material from the apical surface, secrete part of the material into the basolateral milieu in association with chylomicrons. These particles then deliver part of the material to chylomicron target tissues where it might contribute to inflammatory responses. Our findings further support the recent awareness on the role of the gut microflora in whole body inflammation and immunity.▮

REFERENCES

1. Alipour, A., J. W. Elte, H. C. van Zaanen, A. P. Rietveld, and M. C. Cabezas. 2007. Postprandial inflammation and endothelial dysfunction. *Biochem. Soc. Trans.* **35:** 466–469.

2. Burdge, G. C., and P. C. Calder. 2005. Plasma cytokine response during the postprandial period: a potential causal process in vascular disease? *Br. J. Nutr.* **93:** 3–9.

3. Greenberg, A. S., and M. S. Obin. 2006. Obesity and the role of adipose tissue in inflammation and metabolism. *Am. J. Clin. Nutr.* **83:** 461S–465S.

4. Hotamisligil, G. S. 2006. Inflammation and metabolic disorders. *Nature.* **444:** 860–867.

5. Van Gaal, L. F., I. L. Mertens, and C. E. De Block. 2006. Mechanisms linking obesity with cardiovascular disease. *Nature.* **444:** 875–880.

6. Fritsche, K. 2006. Fatty acids as modulators of the immune response. *Annu. Rev. Nutr.* **26:** 45–73.

7. Shi, H., M. V. Kokoeva, K. Inouye, I. Tzameli, H. Yin, and J. S. Flier. 2006. TLR4 links innate immunity and fatty acid-induced insulin resistance. *J. Clin. Invest.* **116:** 3015–3025.

8. Suganami, T., K. Tanimoto-Koyama, J. Nishida, M. Itoh, X. Yuan, S. Mizuarai, H. Kotani, S. Yamaoka, K. Miyake, S. Aoe, et al. 2007. Role of the toll-like receptor 4/NF-kappaB pathway in saturated fatty acid-induced inflammatory changes in the interaction between adipocytes and macrophages. *Arterioscler. Thromb. Vasc. Biol.* **27:** 84–91.

9. Navab, M., S. Y. Hama, S. T. Reddy, C. J. Ng, B. J. Van Lenten, H. Laks, and A. M. Fogelman. 2002. Oxidized lipids as mediators of coronary heart disease. *Curr. Opin. Lipidol.* **13:** 363–372.

10. Navab, M., N. Gharavi, and A. D. Watson. 2008. Inflammation and metabolic disorders. *Curr. Opin. Clin. Nutr. Metab. Care.* **11:** 459–464.

11. Cani, P. D., J. Amar, M. A. Iglesias, M. Poggi, C. Knauf, D. Bastelica, A. M. Neyrinck, F. Fava, K. M. Tuohy, C. Chabo, et al. 2007. Metabolic endotoxemia initiates obesity and insulin resistance. *Diabetes.* **56:** 1761–1772.

12. Erridge, C., T. Attina, C. M. Spickett, and D. J. Webb. 2007. A high-fat meal induces low-grade endotoxemia: evidence of a novel mechanism of postprandial inflammation. *Am. J. Clin. Nutr.* **86:** 1286–1292.

13. Fruchart, J. C., M. C. Nierman, E. S. Stroes, J. J. Kastelein, and P. Duriez. 2004. New risk factors for atherosclerosis and patient risk assessment. *Circulation.* **109:** III15–III19.

14. Botham, K. M., E. H. Moore, C. De Pascale, and F. Bejta. 2007. The induction of macrophage foam cell formation by chylomicron remnants. *Biochem. Soc. Trans.* **35:** 454–458.

15. Vreugdenhil, A. C., C. H. Rousseau, T. Hartung, J. W. Greve, C. van 't Veer, and W. A. Buurman. 2003. Lipopolysaccharide (LPS)-binding protein mediates LPS detoxification by chylomicrons. *J. Immunol.* **170:** 1399–1405.

16. Read, T. E., C. Grunfeld, Z. L. Kumwenda, M. C. Calhoun, J. P. Kane, K. R. Feingold, and J. H. Rapp. 1995. Triglyceride-rich lipoproteins prevent septic death in rats. *J. Exp. Med.* **182:** 267–272.

17. Harris, H. W., C. Grunfeld, K. R. Feingold, T. E. Read, J. P. Kane, A. L. Jones, E. B. Eichbaum, G. F. Bland, and J. H. Rapp. 1993. Chylomicrons alter the fate of endotoxin, decreasing tumor necrosis factor release and preventing death. *J. Clin. Invest.* **91:** 1028–1034.

18. Brun, P., I. Castagliuolo, V. D. Leo, A. Buda, M. Pinzani, G. Palu, and D. Martines. 2007. Increased intestinal permeability in obese mice: new evidence in the pathogenesis of nonalcoholic steatohepatitis. *Am. J. Physiol. Gastrointest. Liver Physiol.* **292:** G518–G525.

19. Kvietys, P. R., R. D. Specian, M. B. Grisham, and P. Tso. 1991. Jejunal mucosal injury and restitution: role of hydrolytic products of food digestion. *Am. J. Physiol. Gastrointest. Liver Physiol.* **261:** G384–G391.

20. Velasquez, O. R., K. Henninger, M. Fowler, P. Tso, and K. D. Crissinger. 1993. Oleic acid-induced mucosal injury in developing piglet intestine. *Am. J. Physiol. Gastrointest. Liver Physiol.* **264:** G576–582.

21. Hathaway, L. J., and J. P. Kraehenbuhl. 2000. The role of M cells in mucosal immunity. *Cell. Mol. Life Sci.* **57:** 323–332.

22. Neal, M. D., C. Leaphart, R. Levy, J. Prince, T. R. Billiar, S. Watkins, J. Li, S. Cetin, H. Ford, and A. Schreiber. 2006. Enterocyte TLR4 mediates phagocytosis and translocation of bacteria across the intestinal barrier. *J. Immunol.* **176:** 3070–3079.

23. Hornef, M. W., T. Frisan, A. Vandewalle, S. Normark, and A. Richter-Dahlfors. 2002. Toll-like receptor 4 resides in the Golgi apparatus and colocalizes with internalized lipopolysaccharide in intestinal epithelial cells. *J. Exp. Med.* **195:** 559–570.

24. Lotz, M., D. Gutle, S. Walther, S. Menard, C. Bogdan, and M. W. Hornef. 2006. Postnatal acquisition of endotoxin tolerance in intestinal epithelial cells. *J. Exp. Med.* **203:** 973–984.

25. Sabesin, S. M., and S. Frase. 1977. Electron microscopic studies of the assembly, intracellular transport, and secretion of chylomicrons by rat intestine. *J. Lipid Res.* **18:** 496–511.

26. Luchoomun, J., and M. M. Hussain. 1999. Assembly and secretion of chylomicrons by differentiated Caco-2 cells. Nascent triglycerides and preformed phospholipids are preferentially used for lipoprotein assembly. *J. Biol. Chem.* **274:** 19565–19572.

27. Xie, Q-M., J-S. Shao, and D. H. Alpers. 1999. Rat intestinal alpha 1-antitrypsin secretion is regulated by triacylglycerol feeding. *Am. J. Physiol. Gastrointest. Liver Physiol.* **276:** G1452–1460.

28. Hunter, R. 1970. Standardization of the chloramine-T method of protein iodination. Proceedings of the Society for Experimental Biology and Medicine. Society for Experimental Biology and Medicine (New York, N.Y.) **133:** 989–992.

29. Ulevitch, R. J. 1978. The preparation and characterization of a radioiodinated bacterial lipopolysaccharide. *Immunochemistry.* **15:** 157–164.

30. Reddy, M. N., and E. J. Fitzsimons. 1986. Clarifying lipemic samples with an air-driven ultracentrifuge for determination of high-density lipoprotein cholesterol. *Clin. Chim. Acta.* **154:** 141–144.

31. Ishibashi, S., S. Perrey, Z. Chen, J. Osuga, M. Shimada, K. Ohashi, K. Harada, Y. Yazaki, and N. Yamada. 1996. Role of the low density lipoprotein (LDL) receptor pathway in the metabolism of chylomicron remnants. A quantitative study in knockout mice lacking the LDL receptor, apolipoprotein E, or both. *J. Biol. Chem.* **271:** 22422–22427.

32. Levin, J., and F. B. Bang. 1964. The role of endotoxin in the extracellular coagulation of limulus blood. *Bull. Johns Hopkins Hosp.* **115:** 265–274.

33. Pool, C., D. F. Nutting, W. J. Simmonds, and P. Tso. 1991. Effect of Pluronic L81, a hydrophobic surfactant, on intestinal mucosal cholesterol homeostasis. *Am. J. Physiol. Gastrointest. Liver Physiol.* **261:** G256–G262.

34. Nayak, N., E. H. Harrison, and M. M. Hussain. 2001. Retinyl ester secretion by intestinal cells: a specific and regulated process dependent on assembly and secretion of chylomicrons. *J. Lipid Res.* **42:** 272–280.

35. Karpe, F., M. Bell, J. Bjorkegren, and A. Hamsten. 1995. Quantification of postprandial triglyceride-rich lipoproteins in healthy men by retinyl ester labeling and simultaneous measurement of apolipoproteins B-48 and B-100. *Arterioscler. Thromb. Vasc. Biol.* **15:** 199–207.

36. Levy, E., M. Mehran, and E. Seidman. 1995. Caco-2 cells as a model for intestinal lipoprotein synthesis and secretion. *FASEB J.* **9:** 626–635.

37. Beatty, W. L., S. Meresse, P. Gounon, J. Davoust, J. Mounier, P. J. Sansonetti, and J. P. Gorvel. 1999. Trafficking of Shigella lipopolysaccharide in polarized intestinal epithelial cells. *J. Cell Biol.* **145:** 689–698.

38. Berg, R. D. 1996. The indigenous gastrointestinal microflora. *Trends Microbiol.* **4:** 430–435.

39. Ravin, H. A., D. Rowley, C. Jenkins, and J. Fine. 1960. On the absorption of bacterial endotoxin from the gastro-intestinal tract of the normal and shocked animal. *J. Exp. Med.* **112:** 783–792.

40. Brenchley, J. M., D. A. Price, T. W. Schacker, T. E. Asher, G. Silvestri, S. Rao, Z. Kazzaz, E. Bornstein, O. Lambotte, D. Altmann, et al. 2006. Microbial translocation is a cause of systemic immune activation in chronic HIV infection. *Nat. Med.* **12:** 1365–1371.

41. Nadhazi, Z., A. Takats, K. Offenmuller, and L. Bertok. 2002. Plasma endotoxin level of healthy donors. *Acta Microbiol. Immunol. Hung.* **49:** 151–157.

42. Adachi, Y., L. E. Moore, B. U. Bradford, W. Gao, and R. G. Thurman. 1995. Antibiotics prevent liver injury in rats following long-term exposure to ethanol. *Gastroenterology.* **108:** 218–224.

43. Caradonna, L., L. Amati, T. Magrone, N. M. Pellegrino, E. Jirillo, and D. Caccavo. 2000. Enteric bacteria, lipopolysaccharides and related cytokines in inflammatory bowel disease: biological and clinical significance. *J. Endotoxin Res.* **6:** 205–214.

44. Wellmann, W., P. C. Fink, F. Benner, and F. W. Schmidt. 1986. Endotoxaemia in active Crohn's disease. Treatment with whole gut irrigation and 5-aminosalicylic acid. *Gut.* **27:** 814–820.

45. Bray, G. A. 2004. Medical consequences of obesity. *J. Clin. Endocrinol. Metab.* **89:** 2583–2589.

46. Tso, P., J. A. Balint, M. B. Bishop, and J. B. Rodgers. 1981. Acute inhibition of intestinal lipid transport by Pluronic L-81 in the rat. *Am. J. Physiol. Gastrointest. Liver Physiol.* **241:** G487–497.

47. Tso, P., J. A. Balint, and J. B. Rodgers. 1980. Effect of hydrophobic surfactant (Pluronic L-81) on lymphatic lipid transport in the rat. *Am. J. Physiol. Gastrointest. Liver Physiol.* **239:** G348–G353.

48. Aspenström-Fagerlund, B., L. Ring, P. Aspenström, J. Tallkvist, N-G. Ilbäck, and A. W. Glynn. 2007. Oleic acid and docosahexaenoic acid cause an increase in the paracellular absorption of hydrophilic compounds in an experimental model of human absorptive enterocytes. *Toxicology.* **237:** 12–23.

49. Abreu, M. T., E. T. Arnold, L. S. Thomas, R. Gonsky, Y. Zhou, B. Hu,

and M. Arditi. 2002. TLR4 and MD-2 expression is regulated by immune-mediated signals in human intestinal epithelial cells. *J. Biol. Chem.* **277:** 20431–20437.

50. Cario, E., I. M. Rosenberg, S. L. Brandwein, P. L. Beck, H. Reinecker, and D. K. Podolsky. 2000. Lipopolysaccharide activates distinct signaling pathways in intestinal epithelial cell lines expressing toll-like receptors. *J. Immunol.* **164:** 966–972.

51. Funda, D. P., L. Tuckova, M. A. Farre, T. Iwase, I. Moro, and H. Tlaskalova-Hogenova. 2001. CD14 Is expressed and released as soluble cd14 by human intestinal epithelial cells in vitro: lipopolysaccharide activation of epithelial cells revisited. *Infect. Immun.* **69:** 3772–3781.

52. Levy, E., D. Menard, I. Suc, E. Delvin, V. Marcil, L. Brissette, L. Thibault, and M. Bendayan. 2004. Ontogeny, immunolocalisation, distribution and function of SR-BI in the human intestine. *J. Cell Sci.* **117:** 327–337.

53. Lobo, M. V., L. Huerta, N. Ruiz-Velasco, E. Teixeiro, P. de la Cueva, A. Celdran, A. Martin-Hidalgo, M. A. Vega, and R. Bragado. 2001. Localization of the lipid receptors CD36 and CLA-1/SR-BI in the human gastrointestinal tract: towards the identification of receptors mediating the intestinal absorption of dietary lipids. *J. Histochem. Cytochem.* **49:** 1253–1260.

54. Cai, S. F., R. J. Kirby, P. N. Howles, and D. Y. Hui. 2001. Differentiation-dependent expression and localization of the class B type I scavenger receptor in intestine. *J. Lipid Res.* **42:** 902–909.

55. Cai, L., E. R. Eckhardt, W. Shi, Z. Zhao, M. Nasser, W. J. De Villiers, and D. R. Van Der Westhuyzen. 2003. Scavenger receptor class B type I reduces cholesterol absorption in cultured enterocyte CaCo-2 cells. *J. Lipid Res.* **45:** 253–262.

56. Vishnyakova, T. G., A. V. Bocharov, I. N. Baranova, Z. Chen, A. T. Remaley, G. Csako, T. L. Eggerman, and A. P. Patterson. 2003. Binding and internalization of lipopolysaccharide by Cla-1, a human orthologue of rodent scavenger receptor B1. *J. Biol. Chem.* **278:** 22771–22780.

57. Cartwright, I. J., D. Plonne, and J. A. Higgins. 2000. Intracellular events in the assembly of chylomicrons in rabbit enterocytes. *J. Lipid Res.* **41:** 1728–1739.

58. Hussain, M. M., S. Fatma, X. Pan, and J. Iqbal. 2005. Intestinal lipoprotein assembly. *Curr. Opin. Lipidol.* **16:** 281–285.

59. Vreugdenhil, A. C. E., A. M. P. Snoek, J. M. Greve, and W. A. Buurman. 2000. Lipopolysaccharide-binding protein is vectorially secreted and transported by cultured intestinal epithelial cells and is present in the intestinal mucus of mice. *J. Immunol.* **165:** 4561–4566.

60. MacDonald, T. T., and G. Monteleone. 2005. Immunity, inflammation, and allergy in the gut. *Science.* **307:** 1920–1925.

61. Huang, J., C. H. Sloop, P. S. Roheim, and L. Wong. 1990. Lipoprotein lipase and hepatic triacylglycerol lipase activities in peripheral and skeletal muscle lymph. *Arterioscler. Thromb. Vasc. Biol.* **10:** 720–726.

62. Mead, J. R., S. A. Irvine, and D. P. Ramji. 2002. Lipoprotein lipase: structure, function, regulation, and role in disease. *J. Mol. Med.* **80:** 753–769.

63. Miura, S., Y. Tsuzuki, R. Hokari, and H. Ishii. 1998. Modulation of intestinal immune system by dietary fat intake: relevance to Crohn's disease. *J. Gastroenterol. Hepatol.* **13:** 1183–1190.