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Emulsified lipids increase endotoxemia: possible role in early postprandial low-grade inflammation☆

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Abstract

Low-grade inflammation is a risk factor for the onset of atherosclerosis. Little is known about the involvement of endotoxin absorption from the gut during the digestion of lipids. In the present study, we first investigated in humans the impact of a mixed meal containing dispersed lipids on postprandial endotoxemia and inflammation. We then investigated the effect of (i) oil emulsification *in vivo* in rats and (ii) fatty acid amounts *in vitro* using Caco-2 cells on postprandial endotoxemia. In humans, postprandial endotoxemia increased early after the meal. Moreover, we evidenced that the endotoxin receptor sCD14 increased during digestion and that chylomicrons could contribute to absorbed endotoxin transport. This could explain the significant peak of inflammatory cytokine IL-6 that we observed 2 h after the mixed meal. Interestingly, in rats, the emulsion led to both higher endotoxemia and hypertriglyceridemia than oil and compared to a control saline load. *In vitro*, incubation of Caco-2 cells with increasing fatty acid concentrations enhanced epithelial absorption of endotoxin. To our knowledge, this is the first study evidencing in healthy humans that, following a mixed meal containing lipids, increased endotoxemia is associated with raised sCD14 and a peak of IL-6. On a repeated basis, this may thus be a triggering cascade for the onset of atherosclerosis. In this respect, optimizing both dietary fat amount and structure could be a possible strategy to limit such low-grade endotoxemia and inflammation by the control of postprandial lipemia.
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1. Introduction

Metabolic diseases of nutritional origin, such as obesity and type 2 diabetes, are characterized by a subclinical inflammatory state that contributes to the development of atherosclerosis [1,2]. Although a correlation between high-fat diet and chronic inflammatory diseases has been shown [3], the specific factors that initiate and maintain low-grade inflammation remain largely unknown. In the case of type 2 diabetes, Creely et al. [4] report the presence in plasma of low doses of proinflammatory bacterial compounds, namely, endotoxins [lipopolysaccharides (LPS)]. Other authors have suggested a link between endotoxemia and atherosclerosis or incident cardiovascular disease events [5,6].

Endotoxins are components of gram-negative bacteria cell wall. Because many gram-negative bacteria are present in the intestinal microflora, representing more than 1 g of LPS being present in the gut [7], intestinal microflora has been suggested as a potential source for these circulating LPS, possibly via translocation [7]. Interestingly, the proportion of gram-negative bacteria in microflora is higher in obese subjects than in lean subjects [8,9]. Furthermore, the reservoir of proinflammatory LPS in the gut might be altered when the diet gets unbalanced [8]. One feature of dietary alterations in obesity and type 2 diabetes is increased fat abundance in the diet, and recent studies report a postprandial inflammation occurring during the digestion of a fat load [10]. Therefore, some authors hypothesized that the low-

Abbreviations: d_{32}, volume–surface mean diameter; CM, chylomicron; LPS, lipopolysaccharides; OA, oleic acid; TAG, triacylglycerol.

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grade inflammation observed in obese subjects could be partly due to the translocation of endogenous LPS from the gut to the blood during digestion of enriched-fat meals [11,12].

In this respect, recent studies investigated the link between high-fat diet and endotoxemia [11,13]. Cani et al. [11] reported that a 4-week high-fat diet in wild-type mice increased plasma endotoxin levels and inflammation in comparison with a control diet. In humans, Amar et al. [13] found a positive correlation between energy intake, fat and endotoxemia. Then, some teams also began to explore the underlying mechanism in mice or humans using high lipid loads [12,14]. Altogether, these results suggest that a chronic fat-rich diet could result in increased endotoxemia and low-grade inflammation due to the repeated endotoxin absorption from the gut during the digestion of lipids, which in turn could increase the risk of atherosclerosis.

However, data are still lacking in humans about the kinetic events linking lipid digestion, endotoxin absorption and postprandial inflammation. Moreover, in the everyday diet, lipids can be of various molecular species (mostly triacylglycerols but also phospholipids) and are incorporated in food products under different physicochemical structures (oil-in-water emulsion such as in numerous high-fat products like ice cream or dressings; water-in-oil emulsion like butter; free oil or dispersed fat inclusions in cheeses or cookies). These various structures and composition of dietary fat products are known to affect the kinetics of lipid absorption [15–18]. We therefore hypothesized that the physicochemical structure of lipids in the food could be one of the determinants of LPS absorption during fat digestion.

In the present study, we thus investigated (i) in healthy young men whether a mixed-meal containing lipids triggers a postprandial endotoxin absorption and inflammation, (ii) in a rodent model whether lipid structure in the food bolus (free oil *vs.* fine emulsion) could change postprandial endotoxin absorption and (iii) in Caco-2 cells whether LPS absorption increases with the amount of dispersed lipids.

2. Materials and methods

2.1. Specific nonpyrogenic material

Care was taken to avoid contamination with exogeneous LPS during experiments: specific single-use nonpyrogenic materials were used, *i.e.*, PS tubes (Becton Dickinson), Axygen tubes (VWR, France) and pyrogen-free pipette tips (Biogenic, Perols, France).

2.2. Subjects

Twelve healthy young men (BMI 24.9±3.6 kg/m^2, age 26.8±6.9 years) were recruited in an ongoing lipid digestion protocol conducted in the Human Nutrition Research Center of Rhône-Alpes. This study was performed in accordance with the principles set out in the Declaration of Helsinki. It has been approved by the Ethics Committee of Lyon under reference no. RG/FL-2005-067 and registered with the health authorities under reference no. DGS 2005/0500. An informed written consent was obtained from the subjects.

On the day of experiment at 0900, subjects took a mixed meal (882 kcal) containing 33 g of fat (291 kcal) and composed of 200 ml of Fortimel (enteral emulsion), 23 g of margarine, 9.4 g of butter, 1 g of olive oil, 85 g of bread, 20 g of jam and 200 g of banana. Blood samples were obtained at baseline and at the following time points: 60, 120 and 240 min after meal. For measurement of plasma LPS, 2 ml of venous blood was obtained from each subject in nonpyrogenic tubes containing 4 μl of injectable heparin (Choay 25,000 IU/ml), under pyrogen-free conditions. Additional blood samples were obtained at the same time to measure sCD14, IL-6 and triacylglycerols. Plasma was collected by centrifugation (10,000 rpm, 10 min, 4°C) and stored at −80°C until analysis. For four of the 12 subjects, plasma samples were obtained in nonpyrogenic tubes, diluted 1:5 in nonpyrogenic water and centrifuged for 4 h at 12,000 rpm at 10°C to separate the chylomicron fraction (CM). Both CM and the remaining fractions were collected and stored at −80°C until endotoxin analysis.

2.3. Animals

All experiments were carried out according to the guidelines laid down by the French Ministère de l'Agriculture and the EU Council Directive for the Care and Use of Laboratory Animals (no. 02889). Male Wistar rats, weighing 200–300 g, were purchased from Harlan (Gannat, France) and kept at 24±1°C on a 12:12-h light/dark cycle (lights on from 0600 to 1800) with a free access to food [65% carbohydrates, 11% fat, 24% proteins (w/w), AO3, 3.2 kcal/g; SAFE, Augy, France] and tap water. Animals were housed individually and adapted to the laboratory conditions for 1 week before the beginning of the experiment.

To permit blood sampling, a catheter was inserted into the left carotid artery. Rats were kept under isoflurane anesthesia, the neck was shaved and the skin was cleaned with a commercial iodine solution (Betadine, Asta Medica, France). A polyethylene catheter (PE-50, biotrol, Villeron, France) was inserted into the left carotid artery. The catheter was exteriorized through an incision in the nape of the neck and filled with heparinized saline (5 mg/L). The wound was sutured (Prolene 4/0, Ethicon) and painted with Betadine. Benzylpenicilline 40,000 IU/kg im (Biclinocilline, Sanofi, France) was injected in the hindpaw to prevent infection. All studies were conducted at least 1 week after surgery to allow animals to overcome the surgical stress and to adapt to the cannulation. Any animal losing weight or showing signs of illness was discarded from the study and immediately euthanised by a pentobarbital overdose (>200 mg/kg).

On the experimental day, rats were randomly assigned to an experimental group (n=5 per group) and received 2.4 ml of one of the three following diets: physiological saline (NaCl 0.9%, Aguettant, Lyon, France); 50% (v/v) sunflower oil (Lesieur)+50% (v/v) physiological saline; or a fine emulsion of sunflower oil in physiological saline (50:50 v/v) using soybean lecithin (35 mg/ml emulsion; Emulpur IP, Cargill, Hamburg, Germany) as emulsifier (homogenizer APV 1000, APV, Evreux, France). Products were nonpyrogenic and of food grade. The particle size distribution of the resulting emulsion was measured by laser light scattering (LS 230, Coulter, Roissy, France). The mean diameter d_{32} and the fat surface area in the sample fed to rats were calculated therefrom as described previously [19].

Rat were fasted overnight prior to the experiments. The experiments were conducted between 0900 and 1500 on awake and unrestrained rats. Blood samples (400 μl) were collected 15 min before forced feeding as baseline value. At Time 0, rats were force fed with a 2.4-ml preparation intragastrically using a syringe equipped with a curved cannula. After forced feeding, rats were contained inside individual glass chambers (1 L) through which air was pumped at a constant flow rate. Blood samples were taken at time points 40, 80, 120, 240 and 360 min for TAG and endotoxin measurements under pyrogen-free conditions on heparin-containing tubes. Plasma was obtained by blood centrifugation (8000×g, 1 min, room temperature), snap frozen in liquid nitrogen and stored at −80°C until measurements.

2.4. Cell culture and treatments

Caco-2/TC7 cells were kindly provided by Dr. D. Lairon (INSERM, U476, Marseille, France) at Passage 62. Cells were seeded on 75-cm^2 flasks (Falcon, Becton Dickinson) until 80–90% confluence. They were routinely cultured in complete DMEM medium (Invitrogen) supplemented with 20% heat-inactivated fetal calf serum (PAA), 1% nonessential amino acids (Invitrogen) and 1% antibiotics (penicillin/streptomycin, Invitrogen), and maintained under a 10% CO_2 atmosphere at 37°C. For experiments, cells were seeded at a density of 1×10^6 cells per filter on microporous (0.4 μm pore size) polyester filters called Transwells (Corning Costar Corp., Cambridge, MA, USA) and grown to confluence in complete medium. The medium was changed in both compartments every 2 days until total differentiation (TC7 cells became confluent and then differentiated approximately 21 days after seeding onto Transwells).

Transepithelial electrical resistance (TEER) was measured every 2 days, prior to media change, using a Millicell-ERS apparatus (Millipore Corp., USA), and on the day of experiments, before and after cell treatments.

Prior to treatment start, cells were incubated with serum-free complete medium for 24 h. In the apical compartment, monolayers were then incubated with 1.5 ml of either of the three types of treatments (LPS alone or LPS coincubated with mixed lipid micelles, containing two different fatty acid concentrations) as described below. The basolateral compartment received 2.5 ml of serum-free complete medium (without antibiotics but complemented with 1% nonessential amino acids, 1% pyruvate and 1% glutamax; Invitrogen).

A stock solution of LPS at 1 mg/ml was prepared using *E. coli* O55:B5 (Sigma, St Louis, MO, USA) and nonpyrogenic water (Biogenic, Perols, France). The incubation medium containing pure LPS at 1 μg/ml was prepared by vortexing 1 μl of stock LPS solution per 1 ml of serum-free complete medium; further dispersion was achieved by standing 5 min in an ultrasonic bath. The incubation media containing mixtures of fatty acid mixed micelles and LPS were prepared as follows. Oleic acid (0.5 or 1.5 mM), 2-oleylglycerol (0.03 mM), soybean lecithin (phosphatidylcholine, 2 mM), cholesterol (0.1 mM), sodium taurocholate (5 mM) and LPS (1 μg/ml) were vortexed in serum-free complete medium and further dispersed in an ultrasonic bath for 5 min at room temperature. All solutions were then maintained at 37°C and used in less than 1 h. All chemicals were purchased from Sigma-Aldrich.

The basolateral media were collected after various incubation times (0.5, 1.5, 3, 6, 24 h) and stored at −80°C in nonpyrogenic tubes for LAL analysis and at −20°C in sterile tubes for lipid analysis.

2.5. Limulus amoebocyte lysate assays

Endotoxemia [endotoxin unit per milliliter (EU/ml)] in plasma, CM and the remaining fraction was determined by using the limulus amoebocyte lysate (LAL) assay

in kinetic chromogenic conditions (Biogenic, Pérols, France). In this method, the time at which the optical density (OD) starts to increase significantly is negatively correlated with the sample endotoxemia.

Briefly, the sample was thawed and diluted 1:10 in pyrogen-free water (Biogenic) and then heated at 70°C for 10 min to inactivate endotoxin-neutralizing agents that inhibit the activity of endotoxin in the LAL assay. Then, the heated sample was subjected to an ultrasonic bath for 5 min and to a 1-min vortex. One hundred microliters of the sample was combined with 100 µl of LAL reagent (Chromolale, Biogenic) in triplicate in pyrogen-free 96-well plates (Biogenic). The raw data (OD and measurement time, collected every 30 s for 200 min) were recovered using the software SkanIt 2.2 for MS Windows for ms 2.2 (Multiscan, Thermo).

In order to avoid any overestimation of endotoxemia due to the inherent sample OD [20,21], the initial sample OD ($OD_{initial}$, average of the values from 0 to 5 min) was first subtracted from all subsequent kinetic OD measurements. According to the standard Biogenic procedure, the increase in OD used in further calculations is 0.1. Therefore, the time for the start of chromogenic reaction (t_{SR}) is identified from the kinetic curve for each well: $t_{SR}=t_{[OD-OD_{initial}=0.1]}$.

A standard curve was prepared in water from *E. coli* O111 endotoxin standard (Biogenic). t_{SR} measured in wells containing the standard curve in water allows to obtain the linear equation for the standard curve: log (endotoxemia)=$a\times$log (t_{SR})+b.

For each studied sample characterized by a specific t_{SR}, and considering the initial 1:10 sample dilution, endotoxemia is thus derived [22]: endotoxemia=10×exp($a\times$log (t_{SR})+b).

The limit of sensitivity of the kinetic chromogenic assay is 0.005 EU/ml; therefore, endotoxemia down to 0.05 EU/ml can be detected in plasma considering a 1:10 dilution.

For Caco-2 cell studies, endotoxins were measured in basolateral media via the LAL assay as described above and using a calibration curve with *E. coli* O111 dispersed in serum-free medium.

2.6. sCD14 Assay

As recently highlighted by Erridge et al. [12], endotoxemia has a fluctuating nature in humans. Depending on individuals and according to innate immunity, many compounds can participate in neutralizing or enhancing endotoxin activity in plasma. Therefore, several authors aimed to use more robust markers of plasma endotoxin exposure than only LPS measurements by LAL assay [12,23–25]. In human plasma, we therefore measured the soluble form of the receptor cluster of differentiation 14 (sCD14) [26,27], which is induced during septic diseases [28] and can also be considered as a marker of endotoxin in plasma [29–31]. Plasma samples were assayed using a sandwich ELISA (Hycult Biotechnology, Netherlands) following the manufacturer's instructions.

2.7. IL-6 Measurements

IL-6 was assayed by cytometric bead array using a commercially available kit (Biorad, France) according to the manufacturer's instructions.

2.8. Plasma triacylglycerol measurements

Plasma triacylglycerol (TAG) was measured with the triglyceride PAP kit (Biomérieux, France) using culture plates (Corning) as described previously [19]. The free glycerol in plasma was measured with the glycerol UV method (R-Biopharm/Boehringer, Mannheim, Germany), so that the real plasma TAG concentration was calculated as: TAG=triglyceride PAP result−glycerol result [19].

2.9. Kinetic parameters

The area under the curve (AUC) and maximum postprandial concentration (C_{max}) were calculated for each subject.

2.10. Immunogold labeling for transmission electron microscopy

Freshly ionized fomvar/carbon-coated 200-mesh nickel grids were placed against a suspension of chylomicron in nonpyrogenic water during 2 min. Nonspecific sites were blocked with 1% BSA in 50 mM Tris-HCl (pH 7.4) for 10 min at RT. Chylomicrons were then incubated with a 1:30 dilution of mouse monoclonal antibody anti-lipopolysaccharide core (*E. coli* and *Enterobacteria*, Hycult Biotechnology, Netherlands) and a 1:6000 dilution of rabbit polyclonal antibody anti-apolipoprotein B (Abcam), in wet chamber, 2 h at 4°C. Grids with suspension were successively washed once in 50 mM Tris-HCl (pH 7.4 and 8.2) at RT. Then, they were incubated in a wet chamber for 45 min at RT in 1% BSA, 50 mM Tris-HCl (pH 8.2) for 20 min at RT, labeled with 10-nm gold conjugated goat anti-rabbit IgG (Tebu-Bio, France) diluted 1:80 in 1% BSA 50 mM Tris-HCl (pH 8.2) and with 20-nm gold conjugated goat anti-mouse IgG (Tebu-Bio, France) diluted 1:80 in 1% BSA 50 mM Tris-HCl (pH 8.2). The grids were successively washed once in 50 mM Tris-HCl (pH 8.2 and 7.4) and in filtrated distilled water. Finally, immunocomplexes were fixed in glutaraldehyde 4% and negatively stained with neutral phosphotungstic acid 1%. Grids were observed on a transmission electron microscope (Jeol 1400JEM, Tokyo, Japan) operating at 80 kV equipped with a Orius 1000 camera and a digital micrograph (Cecil, Faculty of Medicine, Laennec, Lyon, France).

2.11. Statistical analysis

All data are presented as means±S.E.M. and were analysed with Statview 5.0 software (Abacus Concept, Berkeley, CA, USA). Depending on the assumptions of normality, homoscedasticity and sample numbers per group, results were compared either by analysis of variance (ANOVA) for repeated measurements followed by *post*

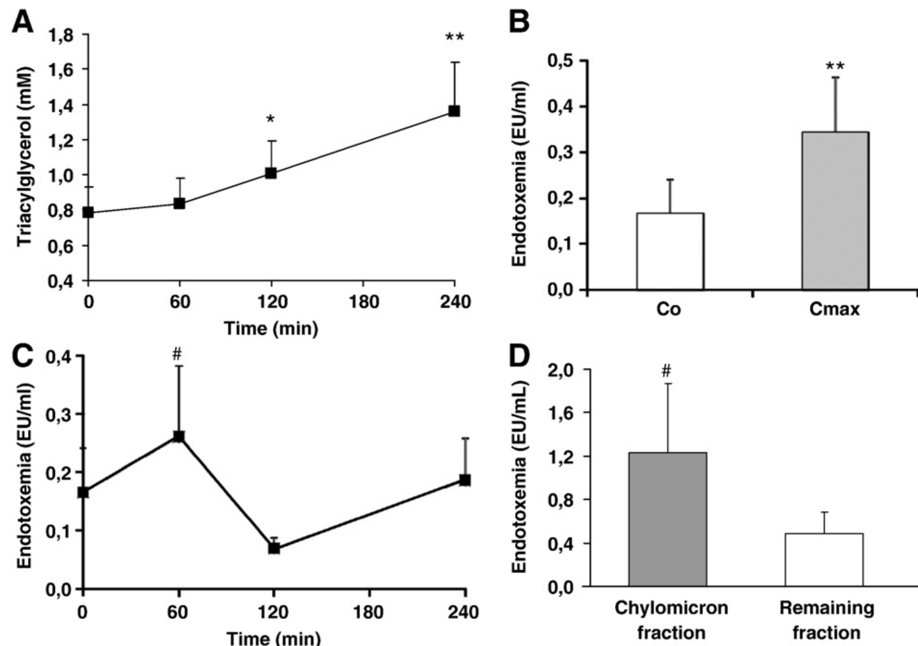

Fig. 1. (A) Kinetics of plasma triacylglycerol (mM) (n=6; *P<.05, **P<.01). (B) Maximum postprandial endotoxemia (C_{max}) *vs.* fasting endotoxemia (Co) (n=12; **P<.01). (C) Kinetics of plasma endotoxemia (EU/ml) (n=12; #P=.057). (D) Endotoxemia in chylomicron and remaining fractions at 60 min (EU/ml) (n=4; #P=.08). Data are means±S.E.M., compared using the paired Student's t test.

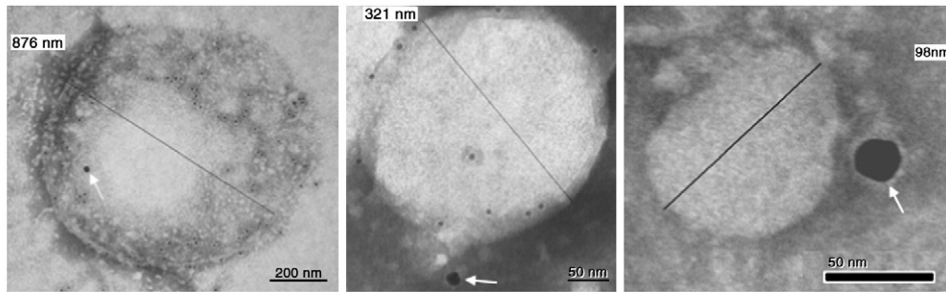

Fig. 2. Immunoelectron microscopy analysis of LPS (*E. coli* and *Enterobacteria* species) and apoB in human chylomicrons. Samples from three different subjects are shown. Bar and the size of chylomicron are noted on each figure. Arrows show gold particles revealing LPS.

hoc test (Fisher PLSD) or by nonparametric Kruskall–Wallis test followed by the Mann–Whitney test. Simple comparisons were performed using Student's *t* test or Mann–Whitney *U* test. Differences were considered significant at the $P<.05$ level.

3. Results

3.1. Kinetics of endotoxemia and inflammation during the digestion of a meal containing emulsified fat in humans

In order to study whether a mixed meal containing dispersed lipids is sufficient to induce a postprandial endotoxemia, we benefited from an ongoing clinical study in which a group of healthy young men took a mixed meal containing 33 g of fat. Then, we evaluated the postprandial evolution of endotoxemia during 4 h. As expected, TAG concentrations in plasma increased during lipid digestion of the test meal as shown in Fig. 1A ($P<.05$ at 120 min, $P<.01$ at 240 min).

As shown in Fig. 1B, the C_{max} of postprandial endotoxemia (0.34±0.12 EU/ml) was significantly higher than fasting endotoxemia (0.17±0.07; $P<.01$). On a kinetic point of view, we observed transient increases in postprandial plasma endotoxin concentration, with a hint of a peak at 60 min on average (0.26±0.04 EU/ml, $P=.057$ compared to baseline) (Fig. 1C). At this peak time, endotoxemia tended to be higher in the CM than in the remaining fraction (Fig. 1D, $P=.08$).

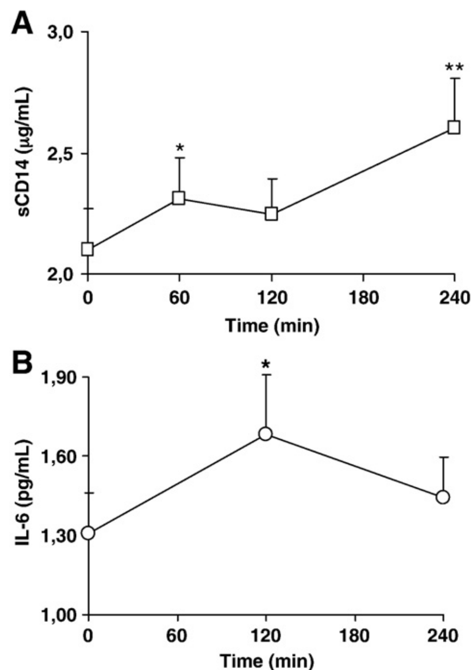

Fig. 3. (A) Kinetics of plasma soluble CD14 (µg/ml) ($n=12$; $^*P<.05$;$^{**}P<.01$). (B) Kinetics of plasma IL-6 (pg/ml) ($n=10$; $^*P=.02$). Data are means±S.E.M., compared using the paired Student's *t* test.

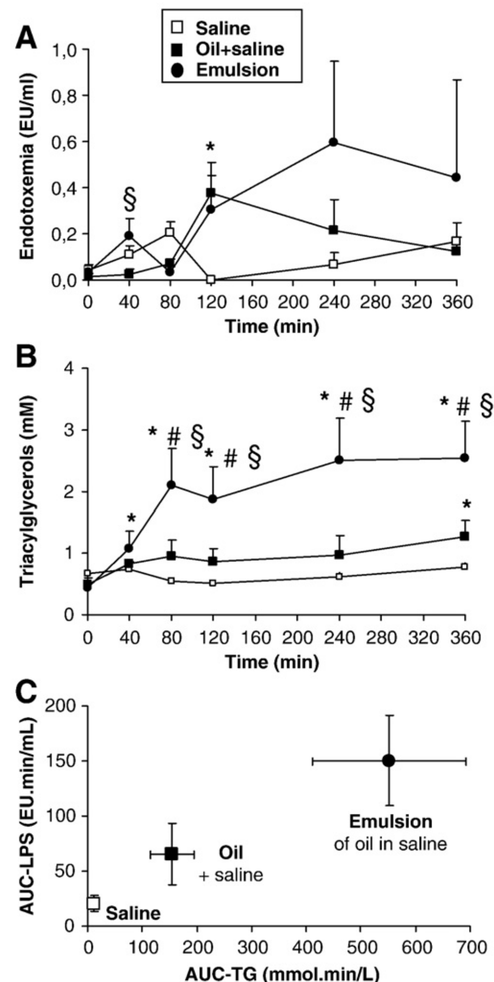

Fig. 4. (A) Kinetics of plasma endotoxemia (EU/ml) in rats after ingestion of saline, oil or emulsion ($n=5$ per group). $^{§}P<.05$, emulsion *vs.* oil; $^{\#}P<.001$, emulsion *vs.* saline; $^*P<.05$, 120 *vs.* 0 min time point for emulsion and oil (ANOVA followed by *post hoc* Fisher PLSD). Data are means±S.E.M. (B) Kinetics of TAG (mM) of rats after feeding saline, oil or emulsion ($n=5$ per group). $^*P<.05$ for Time *t vs.* Time 0, $^{§}P<.05$ emulsion *vs.* oil, $^{\#}P<.001$ emulsion *vs.* saline (ANOVA followed by *post hoc* Fisher PLSD). Data are means±S.E.M. (C) Intergroup correlation between AUC-LPS and AUC-TAG ($n=5$ per group). Data are means±S.E.M.

Table 1
Kinetic parameters after oral administration of physiologic saline, oil and saline, and emulsion of the oil in physiologic saline in rats (n=5 per group)

Parameter	Group		
	Saline	Oil+saline	Emulsion of oil in saline
Endotoxemia			
C_{max} (EU/ml)	0.29±0.04	0.43±0.11	1.37±0.34*
AUC (EU·min/ml)	20.4±7.1	65.5±27.8#	150.3±40.6**
Triacylglycerols			
C_{max} (mmol/L)	0.9±0.1	1.6±0.3 †	2.8±0.6‡, §
AUC (mmol·min/L)	11.6±7.3	154.5±39.9	551.4±140.1‡, §

Data are means±S.E.M. C_{max} indicates maximum concentration. For endotoxemia: *P<.05 emulsion *vs.* saline and oil; **P<.01 emulsion *vs.* saline; #P<.1 oil *vs.* saline. For triacylglycerols: †P<.01 oil *vs.* saline; ‡P<.01 emulsion *vs.* oil; §P<.001 emulsion *vs.* saline (ANOVA followed by *post hoc* Fisher PLSD).

Electron micrographs in Fig. 2 also show that chylomicrons bound LPS from *E. coli* and *Enterobacteria* species. This binding was observed in CM from different subjects independently of the size of CM.

Importantly, we found that soluble endotoxin receptor sCD14 also increased during digestion of the test meal (Fig. 3A). After a transient increase at 60 min, plasma sCD14 reached 2.6±0.2 μg/ml at 240 min (P<.01 compared to fasting sCD14). This reveals a postprandial endotoxin exposure of plasma, consistent with the observed kinetics and C_{max} of LPS.

We examined whether the transient increase of endotoxemia induced postprandial inflammation. As shown in Fig. 3B, we observed a significant increase of IL-6 at 120 min after the meal when compared to baseline value (1.68±0.23 *vs.* 1.31±0.15 pg/ml, P<.05), followed by a decrease at 240 min.

Altogether, these results in healthy humans show that a mixed meal with partly emulsified lipids in different products and of various compositions is able to trigger a postprandial elevation of endotoxins in plasma, together with an early increase of a systemic marker of inflammation, IL-6. We further investigated in a rodent model whether emulsification of lipids can modify such postprandial endotoxemia.

3.2. Impact of fat emulsification on the kinetics of postprandial endotoxemia in rats

We studied the effect of sunflower oil and of a fine emulsion of this oil on endotoxemia and plasma TAG during 360 min. The emulsion presented a droplet size of d_{32}=1.2 μm, *i.e.*, there was 6.2 m^2 of fat/saline interface in the emulsion bolus *vs.* only 0.0005 m^2 in the oil+saline bolus. Therefore, for the same amount of sunflower oil in both preparations, we succeeded in creating dramatically different dispersion states.

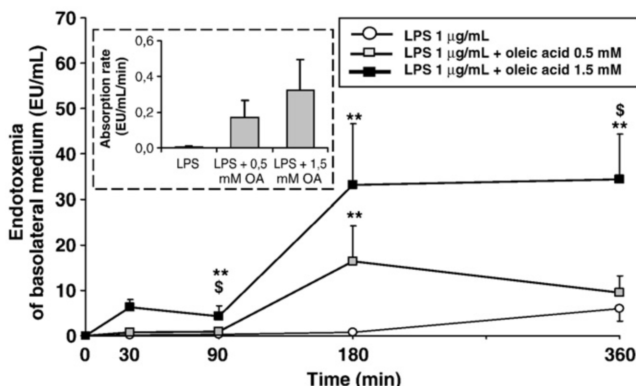

Fig. 5. Endotoxemia in basolateral medium (EU/ml) after Caco-2 cell incubation in the apical medium with either LPS at 1 μg/ml only, LPS at 1 μg/ml+mixed lipid micelles including 0.5 mM oleic acid (OA) or LPS at 1 μg/ml+mixed lipid micelles including 1.5 mM OA (n=6 to 9). Insert represents the LPS absorption rate between 90 and 180 min. **P<.01 *vs.* LPS only; $$P$<.05 between LPS-0.5mM OA and LPS-1.5 mM OA (nonparametric Kruskall–Wallis test followed by a Mann–Whitney test). Data are means±S.E.M.

Endotoxemia rose postprandially after the gastric administration of oil+saline and emulsion preparations (Fig. 4A). Table 1 shows that the highest maximum postprandial concentration (C_{max}) of LPS was observed after oral administration of emulsion (P<.05 *vs.* saline and oil+saline). During the entire postprandial period, the AUC of plasma LPS for the emulsion group was 7.4-fold higher than the AUC of plasma LPS in the saline group (P<.05). A trend (P=.06) was noted for a 2.3-fold higher AUC-LPS in the emulsion group *vs.* the oil+saline group, suggesting that the accumulation of LPS can be more important following the ingestion of emulsified fat.

Furthermore, gavage resulted in an increase of plasma TAG mostly in the emulsion group (Fig. 4B), with the highest C_{max} and AUC of TAG in this group compared to the oil and saline groups (P<.01 and P<.001 respectively; Table 1). Thereby, a correlation was observed in which postprandial LPS accumulation (AUC LPS) was more elevated in a group where lipid absorption was high (AUC TAG) (Fig. 4C).

These data demonstrated in rats that gavage with emulsified oil enhanced postprandial endotoxemia compared to nonemulsified oil. Then, we further investigated *in vitro* the effect of the amount of dispersed lipids on the intestinal bioavailability of LPS.

3.3. Impact of increased concentrations of dispersed fatty acids on LPS absorption by enterocytes in vitro

We studied whether increasing amounts of mixed lipid micelles enhance endotoxin absorption by Caco-2 cells up to 24 h. The integrity of the monolayer was not altered by the treatments (TEER variation <700 Ω cm^2).

Fig. 5 shows that the presence of lipid micelles, compared to incubation with LPS alone, provoked a significant increase of LPS in the basolateral medium (*e.g.*, P<.01 for 1 μg/ml LPS+micelles with 1.5 mM fatty acid compared to 1 μg/ml LPS alone from 3 h onwards). Moreover, LPS in the basolateral medium increased when apical fatty acid concentration was increased from 0.5 to 1.5 mM (P<.05). In this respect, LPS absorption rate after the lag phase (from 90 to 180 min of incubation) increased proportionally with the amount of dispersed fatty acid (insert in Fig. 5). By considering the common conversion factor of 100 pg LPS per EU, we found that the corresponding LPS absorption rate was in the order of magnitude of 0.2% to 0.6% after 6 h of incubation with 0.5 and 1.5 mM fatty acid, respectively.

4. Discussion

Low-grade endotoxemia is increasingly considered as a causal factor in the development of inflammation and atherosclerosis in the context of high-fat diets and obesity [32]. However, the links between fat intake, endotoxemia and low-grade inflammation in the time course of digestion remain poorly characterized. The present work appears to be the first, to our knowledge, to evidence in healthy humans that following a mixed meal containing lipids from different products, a transient increase of endotoxemia is associated with raised sCD14 and an early peak of IL-6. At present, one study reported postprandial endotoxemia in humans after the digestion of a simple fat load (50 g of butter on toast) but failed to observe postprandial inflammation [12]. Moreover, our work is the first to show the kinetics of postprandial endotoxemia in rodents after fat gavage. A previous report in mice failed to observe significant endotoxemia by using a single end-point measurement 30 min after oil gavage [11]. Moreover, our results obtained with Caco-2 cells suggest that this phenomenon may depend on the amount of digested fat. Regarding fat structure, gavage with emulsion significantly increased postpran-

dial endotoxemia in rats (represented by the highest C_{max} and the greatest AUC of LPS) compared to unemulsified oil. This is an important issue because in everyday life, the digestive tract is in contact with various lipid sources.

The higher increase in postprandial lipemia with the emulsion compared to free oil can be partly explained by their difference in fat surface area which is known to enhance lipolysis and postprandial lipemia [33,34]. It may also be due to the presence of phospholipids (lecithin) that are known to increase lipid absorption and chylomicron secretion [17,18,35]. Consistently, previous *in vitro* and *in vivo* studies have also shown that the degree of fat emulsification and the type of emulsifier can impact on digestive lipolysis and digestion kinetics [19,36,37].

Importantly, we showed a link between postprandial endotoxemia and postprandial hypertriglyceridemia when comparing the groups of rats fed with the different diets. This suggests that an intestinal absorption of LPS occurs during absorption of lipid species that are generated in the digestive tract during fat digestion, such as fatty acids and monoacylglycerols [38]. To study such intestinal absorption, Caco-2 cells are recognized as a valuable transport model system for the small intestinal epithelium [39,40] and have been widely used to study absorption of various lipophilic molecules [41–44]. In this respect, our results with Caco-2 cells incubated with LPS show that increased amount of fat in lipolytic products in the apical space, namely, fatty acids, result in increased LPS absorption rate.

Our results obtained *in vivo* suggest that endogenous LPS participate in the concomitant increase of postprandial endotoxemia because no exogenous LPS were added to the food products. Endogenous LPS can originate from gram-negative bacteria found in the gastrointestinal tract [7] and can be internalized by enterocytes [45]. A study in mice has shown, using exogenous radiolabelled LPS, that chylomicron secretion was necessary to the transfer of endotoxin from the enterocyte to the circulation [14]. Because emulsion led to higher postprandial lipemia than oil+saline in rats, the corresponding highest endotoxin accumulation can also be explained by an association between endotoxins and chylomicrons. In this respect, our results show that chylomicrons can also be postprandial carriers for LPS in healthy humans, as revealed by endotoxemia of the CM fraction and electronic microscopy targeted to LPS from *Enterobacteria* and *E. coli*. Chylomicron might thus contribute to the onset of postprandial endotoxemia that we observed in humans and subsequent inflammation.

Interestingly, we observed in humans a postprandial increase of sCD14 during the digestion of a mixed meal. sCD14 is the soluble form of a pattern-recognition receptor that plays a central immunomodulatory role in proinflammatory signaling in response to endotoxins [6]. sCD14 is thus a marker of plasma endotoxin exposure [30,31] that contributes to triggering inflammation and would also be proatherosclerotic [6,46]. We may thus wonder whether observed postprandial sCD14 may contribute to the atherogenic phenotype of postprandial lipemia. Regarding postprandial inflammation, we found a transient increase in plasma IL-6 at 2 h after the mixed meal in humans. In a previous report, CRP and TNF-α did not increase in humans during the digestion of butter [12]. However, our result in humans is consistent with a very recent finding in rats where IL-6 increased in plasma 2 h after a high-saturated fat meal [47]. Our findings in humans and rats suggest that this increase of IL-6 could be due to intestinal endotoxin absorption. Moreover, visceral adipose tissue stands among endocrine organs that are able to secrete IL-6 [48] and it is located at the site of lipid absorption. In humans, recent studies have demonstrated a link between visceral adiposity and the vascular risk associated with obesity [41]. In this respect, visceral fat has been demonstrated to express more inflammatory cytokines than subcutaneous fat in obese states [41]. In rats, NFκB has been shown to be activated postprandially in the visceral adipose tissue 2 h after the high-fat meal [47]. Altogether, our results and the latter study suggest that once absorbed with lipids, LPS could activate CD14 at the surface of immune cells in visceral adipose tissue and induce IL-6 secretion. Further studies are warranted to evidence such a role of endogenous endotoxins in this cascade.

In conclusion, the relationship between fat-rich diets and endotoxemia is an emerging concept, which could explain the onset and maintenance of the subclinical inflammatory state that enhances the development of atherosclerosis. In humans, the present results suggest that the digestion of dispersed dietary lipids can enhance the absorption of endogenous endotoxins, possibly via chylomicrons, and induce transient inflammation. The long-term consequences of such postprandial endotoxemia in the context of high-fat diets in humans, and the underlying mechanisms possibly linked to visceral adipose tissue, remain to be further explored. Moreover, we showed in rats that structured lipid formulations containing phospholipids, such as emulsions, can change the extent of both postprandial lipemia and endotoxemia compared to free oil. Therefore, optimizing the quantity, composition and emulsification state of dietary fats could be a possible strategy to limit endotoxemia by controlling postprandial lipemia, with the aim of preventing low-grade inflammation.

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References

[1] Libby P. Inflammation in atherosclerosis. Nature 2002;420:868–74.
[2] Lepper PM, Schumann C, Triantafilou K, Schneider EM, Triantafilou M, von Eynatten M. Association of lipopolysaccharide-binding protein and coronary artery disease in men. Inflammation Research 2007;56:S154–5.
[3] Greenberg AS, Obin MS. Obesity and the role of adipose tissue in inflammation and metabolism. Am J Clin Nutr 2006;83:461S–5S.
[4] Creely SJ, McTernan PG, Kusminski CM, et al. Lipopolysaccharide activates an innate immune system response in human adipose tissue in obesity and type 2 diabetes. Am J Physiol Endocrinol Metab 2007;292:E740–7.
[5] Pussinen PJ, Tuomisto K, Jousilahti P, Havulinna AS, Sundvall J, Salomaa V. Endotoxemia, immune response to periodontal pathogens, and systemic inflammation associate with incident cardiovascular disease events. Arterioscler Thromb Vasc Biol 2007;27:1433–9.
[6] Stoll LL, Denning GM, Weintraub NL. Potential role of endotoxin as a proinflammatory mediator of atherosclerosis. Arterioscler Thromb Vasc Biol 2004;24:2227–36.
[7] Berg RD. The indigenous gastrointestinal microflora. Trends Microbiol 1996;4:430–5.
[8] Ley RE, Turnbaugh PJ, Klein S, Gordon JI. Microbial ecology — Human gut microbes associated with obesity. Nature 2006;444:1022–3.
[9] Turnbaugh PJ, Ley RE, Mahowald MA, Magrini V, Mardis ER, Gordon JI. An obesity-associated gut microbiome with increased capacity for energy harvest. Nature 2006;444:1027–31.
[10] Lundman P, Boquist S, Samnegard A, et al. A high-fat meat is accompanied by increased plasma interleukin-6 concentrations. Nutr Metab Cardiovasc Dis 2007;17:195–202.
[11] Cani PD, Amar J, Iglesias MA, et al. Metabolic endotoxemia initiates obesity and insulin resistance. Diabetes 2007;56:1761–72.
[12] Erridge C, Attina T, Spickett CM, Webb DJ. A high-fat meal induces low-grade endotoxemia: evidence of a novel mechanism of postprandial inflammation. Am J Clin Nutr 2007;86:1286–92.
[13] Amar J, Burcelin R, Ruidavets JB, et al. Energy intake is associated with endotoxemia in apparently healthy men. Am J Clin Nutr 2008;87:1219–23.

[14] Ghoshal S, Witta J, Zhong J, de Villiers W, Eckhardt E. Chylomicrons promote intestinal absorption of lipopolysaccharides. J Lipid Res 2009;50:90–7.
[15] Michalski MC. Specific molecular and colloidal structures of milk fat affecting lipolysis, absorption and postprandial lipemia. Eur J Lipid Sci Technol 2009;111: 413–31.
[16] Singh H, Ye A, Horne D. Structuring food emulsions in the gastrointestinal tract to modify lipid digestion. Prog Lipid Res 2009;48:92–100.
[17] Verger R. Enzyme kinetics of lipolysis. Methods Enzymol 1980;64:340–92.
[18] Parlier RD, Frase S, Mansbach II CM. Intraenterocyte distribution of absorbed lipid and effects of phosphatidylcholine. Am J Physiol 1989;256:G349–355.
[19] Michalski MC, Soares AF, Lopez C, Leconte N, Briard V, Geloen A. The supramolecular structure of milk fat influences plasma triacylglycerols and fatty acid profile in the rat. Eur J Nutr 2006;45:215–24.
[20] Erridge C, Spickett CM, Attina T, Webb DJ. Triglyceride concentrations and endotoxemia — Reply to J Rood and SR Smith. Am J Clin Nutr 2008;88:249–50.
[21] Rood J, Smith SR. Triglyceride concentrations and endotoxemia. Am J Clin Nutr 2008;88:248–9.
[22] Bode C, Kugler V, Bode JC. Endotoxemia in patients with alcoholic and nonalcoholic cirrhosis and in subjects with no evidence of chronic liver-disease following acute alcohol excess. J Hepatol 1987;4:8–14.
[23] Grunwald U, Kruger C, Schutt C. Endotoxin-neutralizing capacity of soluble CD14 is a highly conserved specific function. Circ Shock 1993;39:220–5.
[24] Bolke E, Jehle PM, Storck M, Nothnagel B, Stanescu A, Orth K. Endotoxin release and endotoxin neutralizing capacity during colonoscopy. Clin Chim Acta 2001; 303:49–53.
[25] Schiffrin EJ, Parlesak A, Bode C, et al. Probiotic yogurt in the elderly with intestinal bacterial overgrowth: endotoxaemia and innate immune functions. Br J Nutr 2009;101:961–6.
[26] Bazil V, Strominger JL. Shedding as a mechanism of down-modulation of CD14 on stimulated human monocytes. J Immunol 1991;147:1567–74.
[27] Wright SD. Multiple receptors for endotoxin. Curr Opin Immunol 1991;3:83–90.
[28] Burgmann H, Winkler S, Locker GJ, et al. Increased serum concentration of soluble CD14 is a prognostic marker in gram-positive sepsis. Clin Immunol Immunopathol 1996;80:307–10.
[29] Hiki N, Berger D, Dentener MA, et al. Changes in endotoxin-binding proteins during major elective surgery: important role for soluble CD14 in regulation of biological activity of systemic endotoxin. Clin Diagn Lab Immunol 1999;6:844–50.
[30] Bas S, Gauthier BR, Spenato U, Stingelin S, Gabay C. CD14 is an acute-phase protein. J Immunol 2004;172:4470–9.
[31] Yao YM, Yu Y, Fang WH, Zhai HX, Shi ZG. The significance of lipopolysaccharide-binding protein and CD14 in increasing sensitivity to endotoxin response and multiple organ dysfunction syndrome following trauma. Crit Care Med 1999;27:A131.
[32] Cani PD, Delzenne NM. The role of the gut microbiota in energy metabolism and metabolic disease. Curr Pharm Des 2009;15:1546–58.
[33] Armand M, Pasquier B, Andre M, et al. Digestion and absorption of 2 fat emulsions with different droplet sizes in the human digestive tract. Am J Clin Nutr 1999;70: 1096–106.
[34] Garaiova I, Guschina I, Plummer S, Tang J, Wang D, Plummer N. A randomised cross-over trial in healthy adults indicating improved absorption of omega-3 fatty acids by pre-emulsification. Nutrition Journal 2007;6:4.
[35] Nishimukai M, Hara H, Aoyama Y. Enteral administration of soybean lecithin enhanced lymphatic absorption of triacylglycerol in rats. Br J Nutr 2003;90: 565–71.
[36] Michalski MC, Briard V, Desage M, Geloen A. The dispersion state of milk fat influences triglyceride metabolism in the rat —A (CO_2)-C-13 breath test study. Eur J Nutr 2005;44:436–44.
[37] Mun S, Decker EA, McClements DJ. Influence of emulsifier type on in vitro digestibility of lipid droplets by pancreatic lipase. Food Res Int 2007;40:770–81.
[38] Carriere F, Renou C, Lopez V, et al. The specific activities of human digestive lipases measured from the in vivo and in vitro lipolysis of test meals. Gastroenterology 2000;119:949–60.
[39] Hidalgo IJ, Raub TJ, Borchardt RT. Characterization of the Human-colon carcinoma cell-line (Caco-2) as a model system for intestinal epithelial permeability. Gastroenterology 1989;96:736–49.
[40] Levy E, Mehran M, Seidman E. Caco-2 cells as a model for intestinal lipoprotein synthesis and secretion. FASEB J 1995;9:626–35.
[41] Garrett DA, Failla ML, Sarama RJ. Development of an in vitro digestion method to assess carotenoid bioavailability from meals. J Agric Food Chem 1999;47:4301–9.
[42] Garrett DA, Failla ML, Sarama RJ. Estimation of carotenoid bioavailability from fresh stir-fried vegetables using an in vitro digestion/Caco-2 cell culture model. J Nutr Biochem 2000;11:574–80.
[43] Reboul E, Abou L, Mikail C, et al. Lutein transport by Caco-2 TC-7 cells occurs partly by a facilitated process involving the scavenger receptor class B type I (SR-BI). Biochemical Journal 2005;387:455–61.
[44] Peretti N, Delvin E, Sinnett D, Marcil V, Garofalo C, Levy E. Asymmetrical regulation of scavenger receptor class B type I by apical and basolateral stimuli using Caco-2 cells. J Cell Biochem 2007;100:421–33.
[45] Hornef MW, Frisan T, Vandewalle A, Normark S, Richter-Dahlfors A. Toll-like receptor 4 resides in the Golgi apparatus and colocalizes with internalized lipopolysaccharide in intestinal epithelial cells. J Exp Med 2002;195:559–70.
[46] Amar J, Ruidavets JB, Sollier CBD, et al. Soluble CD14 and aortic stiffness in a population-based study. J Hypertens 2003;21:1869–77.
[47] Magné J, Mariotti F, Fischer R, Mathé V, Tomé D, Huneau JF. Early postprandial low-grade inflammation after high-fat meal in healthy rats: possible involvement of visceral adipose tissue. J Nutr Biochem 2009 [ePub ahead of press].
[48] Mohamed-Ali V, Goodrick S, Rawesh A, et al. Subcutaneous adipose tissue releases interleukin-6, but not tumor necrosis factor-alpha, in vivo. Journal of Clinical Endocrinology & Metabolism 1997;82:4196–200.