Propensity to high-fat diet-induced obesity in rats is associated with changes in the gut microbiota and gut inflammation

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Barbier de La Serre C, Ellis CL, Lee J, Hartman AL, Rutledge JC, Raybould HE. Propensity to high-fat diet-induced obesity in rats is associated with changes in the gut microbiota and gut inflammation. Am J Physiol Gastrointest Liver Physiol 299: G440–G448, 2010. First published May 27, 2010; doi:10.1152/ajpgi.00098.2010.—Consumption of diets high in fat and calories leads to hyperphagia and obesity, which is associated with chronic “low-grade” systemic inflammation. Ingestion of a high-fat diet alters the gut microbiota, pointing to a possible role in the development of obesity. The present study used Sprague-Dawley rats that, when fed a high-fat diet, exhibit either an obesity-prone (DIO-P) or obesity-resistant (DIO-R) phenotype, to determine whether changes in gut epithelial function and microbiota are diet or obese associated. Food intake and body weight were monitored daily in rats maintained on either low- or high-fat diets. After 8 or 12 wk, tissue was removed to determine adiposity and gut epithelial function and to analyze the gut microbiota using PCR. DIO-P but not DIO-R rats exhibit an increase in toll-like receptor (TLR4) activation associated with ileal inflammation and a decrease in intestinal alkaline phosphatase, a luminal enzyme that detoxifies lipopolysaccharide (LPS). Intestinal permeability and plasma LPS were increased together with phosphorylation of myosin light chain and localization of occludin in the cytoplasm of epithelial cells. Measurement of bacterial 16S rRNA showed a decrease in total bacterial density and an increase in the relative proportion of Bacteroidales and Clostridiales orders in high-fat-fed rats regardless of phenotype; an increase in Enterobacteriales was seen in the microbiota of DIO-P rats only. Consumption of a high-fat diet induces changes in the gut microbiota, but it is the development of inflammation that is associated with the appearance of hyperphagia and an obese phenotype.

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gain insight into whether changes in the gut microbiota are driven by the HF diet or by the ensuing obesity.

In the present study, the hypothesis to be tested was that ingestion of a HF diet alters the gut microbiota, induces gut inflammation and elevated LPS, and that this only occurs in obese-prone rats. To test this hypothesis, rats were maintained on a HF diet or low-fat control diet for 8 or 12 wk, and gut inflammation, tight junction proteins, intestinal permeability, and intestinal alkaline phosphatase activity (IAP), a duodenal brush-border enzyme that can detoxify LPS, were measured. In addition, differences at the phylogenetic order level in both dominant and minor bacterial orders were determined in the cecal contents. Previous data in humans and rodents have shown that >90% of the microbiota in a normal distal gut is represented by the Bacteroidetes and Firmicutes phyla (21). Among the Bacteroidetes, the Bacteroides class and Bacteroidales order dominate, and, among the Firmicutes, the Clostridiales class and Clostridiaceae order dominate; moreover, members of these phyla have been shown to be influenced by high-fat feeding or obesity (3, 28, 38–39). An increase in the γ-proteobacteria class, in which belong the order Enterobacteriales and the family Enterobacteriaceae, has also been reported on a HF diet (21). Host inflammation has also been shown to promote the growth of aerotolerant bacteria such as Enterobacteriaceae (30). Therefore Bacteroidales, Clostridiales, and Enterobacteriales were chosen for the current study. The data showed that consumption of a HF diet induces changes in gut microbiota, but it is the development of inflammation in response to these changes that is associated with the appearance of hyperphagia and an obese phenotype.

MATERIALS AND METHODS

Animals. Male Sprague-Dawley rats (initial body wt = 262 ± 2 g) were single-housed in a temperature-controlled room with a 12:12-h light-dark cycle and fed either a low-fat diet (LF; Research Diets 12450B) or a HF diet (Research Diets 12451) for 8 or 12 wk. The diets provided 3.85 kcal/g of energy for the LF [70% carbohydrate, 20% protein, 10% fat [saturated fatty acids (SAT), 25.1%; monounsaturated fatty acids (MUFA), 34.7%; polyunsaturated fatty acids (PUFA), 40.2%]] and 4.73 kcal/g of energy for the HF [35% carbohydrate, 20% protein, 45% fat (SAT, 34.7%; MUFA, 34.7%; PUFA, 34.7%)]. Body weight and food intake were recorded daily. Animals were killed after 8 wk (n = 17; LF n = 6, HF = 11) or 12 wk (n = 13; LF n = 5, HF = 8). An additional group of rats (n = 12; LF n = 4, HF = 8) were fed diets for 12 wk, and body weight was recorded weekly. All experiments were performed in accordance with protocols reviewed and approved by the Institutional Animal Care and Use Committee, University of California Davis.

Measurement of gut permeability in vivo. After 10 wk on the diet, rats were fasted for 6 h and gavaged with 4,000 kDa FITC-labeled dextran diluted in saline (Sigma-Aldrich, St. Louis, MO) (500 mg/kg, 125 mg/ml). After 1 h, blood (500 μl) was collected from the tail vein and centrifuged (10,000 rpm for 3 min at 4°C), and FITC-dextran diluted in saline (Sigma-Aldrich, St. Louis, MO) (500 mg/kg, 125 mg/ml) was gavaged. Blood was collected by cardiac puncture and centrifuged, and plasma was stored at −80°C. Duodenal and ileal tissues and mucosa were collected and stored at −80°C.

Measurement of myeloperoxidase activity. Myeloperoxidase (MPO) activity was determined as a measure of inflammation and neutrophil infiltration using an o-dianisidine assay (7, 26, 32). Ileal samples were sonicated over ice for 20 s in 500 μl of 0.5% hexadecyltrimethylammonium bromide in potassium phosphate buffer (pH 6). Samples were frozen and thawed three times, sonicated for 10 s, and centrifuged (10,000 rpm, 30 min, 4°C), and the pellet was frozen and thawed; this cycle was repeated two times. Final supernatant (10 μl)
was mixed with 290 μl of potassium phosphate buffer containing 0.167 mg/ml of o-dianisidine dihydrochloride and 0.005% of hydrogen peroxide. Absorbance was read at 450 nm at 5 min (32). Activity was expressed as the difference between the absorbance after a 5-min reaction time and the baseline absorbance per milliliter of supernatant and per milligram of tissues.

Measurement of IAP activity. IAP activity was measured with a SensoLyte p-nitrophenyl phosphate (pNPP) Alkaline Phosphatase Assay Kit (no. 71230; Anaspec, Fremont, CA) according to recommendations of the manufacturer. Briefly, duodenal tissue was homogenized with lysis buffer (400 μl/50 mg tissue) and centrifuged (15 min, 10,000 rpm, 4°C), and the supernatant was diluted 1:200. Samples were incubated with pNPP reaction mixture for 20 min, and absorbance was read at 405 nm.

Measurement of plasma LPS. LPS was measured with a Pyrochrome Lysate Mix, a quantitative chromogenic reagent (Associate of Cape Cod, East Falmouth, MA), diluted in Glucashield buffer (Associate of Cape Cod) which inhibits cross-reactivity with (1→3)-β-D-}

Fig. 2. A: myeloperoxidase (MPO) activity in the ileum in LF, DIO-R, and DIO-P rats after 12 wk on respective diets. There was a significantly higher level of MPO activity in DIO-P rats compared with LF or DIO-R animals (DIO-P vs. LF, *P* < 0.01, DIO-P vs. DIO-R, *P* < 0.05). B: plasma levels of lipopolysaccharide (LPS) were increased in DIO-P but not DIO-R rats after 12 wk on a HF diet (DIO-P vs. LF or DIO-R, *P* < 0.05). C and D; photomicrographs of toll-like receptor (TLR)4/MD2 complex immunoreactivity in transverse sections of ileal crypts from LF, DIO-R, and DIO-P rats after 12 wk on respective diets. E: quantification of TLR4/MD2 immunoreactivity expressed as a percentage of positive pixels from 4–6 images/rat; *n* = 4/group. There was more immunoreactivity in DIO-P than LF and DIO-R tissue (DIO-P vs. LF or DIO-R, *P* < 0.05). Different letters denote significant differences between groups.
glucans. Briefly, plasma samples were diluted 1:10 in 10 mM MgCl₂ (Sigma-Aldrich) in pyrogen-free water (Lonza, Basel, Switzerland) and heated for 10 min at 70°C. Samples and reactive solution were incubated at 37°C for 50 min, and absorbance was read at 405 nm.

Immunochemical localization of TLR4/MD2 complex and occludin. Cryostat sections (4 μm) of ileum were fixed (1% paraformaldehyde, 15 min) and washed in PBS. Nonspecific background was blocked by 30 min incubation at 37°C with 20% goat serum. Slices were incubated with primary antibodies [affinity-purified anti-mouse TLR4/MD2 1:100, no. 14–9924 (eBioscience, San Diego, CA) or mouse anti-occludin 1:200, no. 33–1500 (Invitrogen, Carlsbad, CA)] for 2.5 h at 37°C, washed three times in PBS, and incubated with secondary antibody (Alexa Fluor goat anti-mouse 488 1:200; Invitrogen). For immunolocalization of TLR4/MD2, confocal images were obtained (Radiance system 2100; Bio-Rad Labs, Hercules, CA) and for immunolocalization of occludin fluorescent images were obtained with an Olympus Provis AX70 microscope (Olympus, Melville, NY); images were analyzed with Scion Image software (Scion, Frederick, MD).

Measurement of phosphorylated myosin light chain by Western blot. Phosphorylated myosin light chain (p-MLC) expression was measured by Western blot of ileum as previously described (17). Briefly, 30 g of proteins were used, samples were loaded in precast 7% Tris acetate gel, and the gel was run for 60 min at 125 volts (Invitrogen Power Case 500). The proteins were transferred (1 h at 30 V, 220 mA) from the gel to a Fluorotrans polyvinylidene difluoride membrane ( Pall, East Hills, NY). Skim milk (10%) in TBS-Tween 20 (TBST) was used to block. Primary antibody dilution was 10 μl in 10% milk-TBST [glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (14C10) rabbit monoclonal antibody, no. 2118 (Cell Signaling, Beverly, MA); p-MLC (18Thr)-R, no. sc-19848-R (Santa Cruz Biotechnology)]. Milk-TBST (10 μl in 25%) of secondary antibody [anti-rabbit IgG horseradish peroxidase-linked, no. 7074 (Cell Signaling), were used as well as horseradish peroxidase (1:2,000; Cell Signaling). The membrane was exposed to film for 30 min and developed in a 100 Plus automatic X-Ray film Processor (All Pro Imaging, Hicksville, NJ). The film was analyzed by Imagequant version 5.1 software (Amersham, Biosciences, Amersham, UK) (17).

Quantitative assessment of gut microbiota order abundance by sequence analysis of the microbial 16S rRNA gene. The materials and methods are described in detail [see supplemental information and Ref. 20 (Supplemental data for this article may be found on the American Journal of Physiology: Gastrointestinal and Liver Physiology website.)]. Cecal contents were removed and maintained on dry ice for same-day processing by phenol-chloroform-sodium acetate-ethanol- and bead beating-based cellular lysis and nucleic acid isolation/purification methods. Each quantitative PCR (qPCR) well was run in triplicate and contained 10μl of 2× Takara Perfect Real Time master mix (7.2 μl of water, 0.8 μl of a 10 μM F/R primer mix, 2 μl of either an optimized dilution of 1:500 of extracted template DNA in DNase/RNase-free water for specimen analysis or a serial dilution
Effect of a HF diet on body weight, adiposity, and food intake. After 8 wk on a HF diet, two different phenotypes emerged within the HF group; animals with the highest body weight were assigned to the DIO-P group, the other rats were designated DIO-R (8 wk: DIO-R n = 5, DIO-P n = 6; 12 wk: DIO-R n = 4, DIO-P n = 4). DIO-P rats had a significantly higher body weight compared with LF controls (457 ± 5 vs. 418 ± 8 g, P < 0.001; Fig. 1A) and with DIO-R (457 ± 5 vs. 427 ± 4 g, P < 0.01). After 4 wk on a HF diet, DIO-P animals had a higher caloric intake than LF and DIO-R rats (DIO-P vs. LF, P < 0.001, DIO-P vs. DIO-R, P < 0.001; week 7: DIO-P vs. LF, P < 0.001, DIO-P vs. DIO-R, P < 0.01; week 10: DIO-P vs. LF, P < 0.001, DIO-P vs. DIO-R, P < 0.001; Fig. 1B). After either 8 or 12 wk on a HF diet, DIO-P rats had a significantly higher adiposity index than the LF and DIO-R rats (week 8: P < 0.01, week 12: P < 0.001); there was no significant difference in adiposity between DIO-R and LF-fed rats (Fig. 1C).

Effect of a HF diet on intestinal MPO, TLR4 activation, and plasma levels of LPS. After 12 wk on a HF diet, there was a significantly higher MPO activity in ileal mucosa from DIO-P compared with either LF or DIO-R rats (DIO-P vs. LF, P < 0.01, DIO-P vs. DIO-R, P < 0.05; Fig. 2A). DIO-P rats exhibited higher LPS concentration in plasma than LF and DIO-R animals (DIO-P vs. LF or DIO-R, P < 0.05; Fig. 2B).

TLR4 activation was measured in ileal epithelium using immunolocalization of the TLR4/MD2 complex. In LF-fed and DIO-R rats, immunoreactivity was localized to the apical region of enterocytes. However, in the ileum of DIO-P rats, immunoreactivity was localized within and along the basolateral region of enterocytes. Similar translocation has been shown in vitro on T84 human epithelial cells when exposed to LPS (14). Quantification showed a significantly higher immunoreactivity for the TLR4/MD2 complex in DIO-P rats compared with LF and DIO-R animals (%labeled pixels, P < 0.05) (Fig. 2, C–E).

Effect of a HF diet on intestinal IAP. IAP activity was measured in duodenal mucosa after 8 wk on the diets. There was a decrease in IAP activity in DIO-P rats compared with LF and DIO-R animals (DIO-P vs. LF, P < 0.05, DIO-P vs. DIO-R, P < 0.01; Fig. 3). There was no apparent difference in individuals within the LF-fed group, and the distribution was normal.

Effect of a HF diet on right junction-associated proteins and intestinal permeability. p-MLC expression was measured by Western blot in the ileum after 12 wk on the diets and found to be increased in DIO-P rats compared with LF and DIO-R rats (DIO-P vs. LF, P < 0.001). Different letters denote significant differences between groups.

RESULTS

Effect of a HF diet on intestinal permeability. Permeability was measured by appearance of FITC-labeled dextran in plasma. After either 8 or 12 wk on a HF diet, DIO-P rats had a significantly higher gut permeability compared with LF and DIO-R animals (DIO-P vs. LF, P < 0.001). DIO-R rats also exhibited a significant increase in gut permeability compared with the LF control group (DIO-R vs. LF, P < 0.001). Different letters denote significant differences between groups.
significant increase in p-MLC expression in DIO-P rats compared with LF and DIO-R animals (DIO-P vs. LF, \( P < 0.01 \), DIO-P vs. DIO-R, \( P < 0.05 \); Fig. 4, A and B).

Occludin translocation was quantified as the percentage of positive pixels in the cytoplasm (Fig. 5, A and B). In sections of ileum from LF or DIO-R rats, occludin immunoreactivity was located in the region of the tight junctions. There was an increase in immunoreactive occludin in the cytoplasm in DIO-P rats compared with LF and DIO-R animals (DIO-P vs. LF or DIO-R, \( P < 0.001 \)).

As a consequence of intestinal inflammation, epithelial barrier integrity was altered in DIO-P rats. There was a significant increase in gut permeability, measured in vivo by appearance of FITC-labeled dextran in plasma, in DIO-P rats compared...
with LF and DIO-R animals after 10 wk on respective diets (DIO-P vs. LF or DIO-R, P < 0.001; Fig. 6).

**Effect of a HF diet on gut microbiota composition.** Cecal samples were taken from rats maintained for 8 wk on respective diets (LF = 16, DIO-R = 10, DIO-P = 10). Total bacterial 16S rRNA gene copies and relative order abundances were determined by qPCR. From the first quantitatively dominant phylum, the Firmicutes (Gram-positive), the abundance of dominant Clostridiales order was determined. From the second quantitatively dominant phylum, the Bacteroidetes (Gram-negative), the abundance of dominant Bacteroidales order was determined. In addition, from the quantitatively minor phylum, the Proteobacteria (Gram-negative), the abundance of Enterobacteriales order was determined (15).

There was no difference between LF, DIO-R, and DIO-P animals regarding the number of total bacterial 16S rRNA gene copies per gram of wet weight of effluent. However, there was a decrease in total bacteria in HF- compared with LF-fed rats (LF vs. HF, P < 0.05; Fig. 7A). Similarly, there was no difference between LF, DIO-R, and DIO-P animals in the relative abundances of Bacteroidales (expressed as a percentage of total bacteria). However, there was a significant increase in Bacteroidales in HF- compared with LF-fed rats (LF vs. HF, P < 0.01; Fig. 7B). Moreover, ingestion of a HF diet was associated with an increase in Clostridiales compared with a LF diet regardless of propensity for obesity (LF vs. DIO-R, P < 0.05, LF vs. DIO-P, P < 0.01; Fig. 7C). There was, however, a marked and significant difference in the relative abundance of Enterobacteriales in the DIO-P animals compared with either DIO-R or LF-fed animals (DIO-P vs. LF or DIO-R, P < 0.05; Fig. 7D).

**DISCUSSION**

In the present study, we sought to investigate the role of gastrointestinal inflammation, changes in intestinal permeability, LPS, and the gut microbiota in propensity of the obesigenic effects of HF diets. As previously described by us and others (27, 34), Sprague-Dawley rats express two distinct phenotypes in response to high-fat feeding; DIO-P rats only show an increase in body weight and adiposity and become hyperphagic. In the present study, we show for the first time that only obese-prone rats exhibit ileal inflammation. In these obese rats, there is a decrease in IAP activity and an increase in TLR4 activation in the gut wall. Activation of TLR4 has previously been shown to alter tight junctions and increase intestinal permeability (24); we show an alteration in tight-junction proteins with an increase in p-MLC and an altered cellular distribution of occludin. Rats resistant to obesity do not exhibit intestinal epithelial barrier alterations, changes in gut permeability, or increase in plasma LPS. Taken together, these data suggest that activation of the TLR4, gut inflammation, and LPS plays a major role in the development of the obese phenotype in response to ingestion of high-fat, high-calorie foods.

Because our data point to a role for the bacterial product LPS, we also determined changes in the gut microbiota in DIO-P and DIO-R animals. High-fat feeding induced a significant decrease in the total bacterial count and an increase in the relative proportion of Bacteroidales and Clostridiales; however, these changes were independent of the obese or lean phenotype. This suggests that high-fat feeding induces a change in the gut microbiota but that this is not always associated with obesity, calling into question the concept of an “obese” microbiota. These data are in agreement with that published recently using mice null for RELMβ, a goblet cell-specific gene whose expression in dependent in gut microbiota and involved in Th2 cytokine-immune signaling that renders mice susceptible to intestinal inflammation (21). Female RELMβ null mice are relatively resistant to the obesigenic effects of a HF diet, yet similar changes in the gut microbiota were seen in both null and wild-type mice in response to HF diet, suggesting that diet rather than the obese phenotype determines the composition of the gut microbiota. In the current study, the ability to maintain Sprague-Dawley rats in the same facility on the identical HF diet has enabled us to differentiate the diet from the obese phenotype in inducing changes in the microbiota. Moreover, DIO-R rats maintain the same body weight, food intake, and adiposity as those ingesting LF diet, providing a better control for the effects of diet alone.

There was, however, a significant increase in the Enterobacteriales in the diet-induced obese rats; a bloom in Proteobacteria was also observed in the study of Hildebrandt et al. (21) but was seen in both wild-type mice and the relatively lean RELMβ mice. However, because the knockout mice are only relatively resistant to the obesigenic effects of the HF diet, unlike DIO-R in the current study, it is difficult to compare the data between the two studies. An increase in Enterobacteraceae family within this order has been associated with gut inflammation; induction of experimental colitis in rodents is followed by an increase in this family, suggesting that it may be a consequence of gut inflammation rather than a cause (30). In the context of the current study, the presence of gut inflammation in the diet-induced obese rats as evidenced by an increase in MPO and activation of the TLR4 suggests that the increase in Enterobacteriales is secondary to the inflammation.

IAP is a duodenal brush-border enzyme that is secreted in the duodenal lumen and is able to detoxify LPS by dephosphorylation of the toxic lipid A region (19). IAP secretion has been reported to increase after fat feeding (1) and LPS stimu-
lation (25). In zebra fish, endogenous IAP has been shown to have a protective role against LPS toxicity (6). The reason for the different expression of IAP in response to the HF diets in some rats is not clear, but IAP has been associated with reduced LPS-induced inflammation (18). Interestingly, IAP null mice gain more weight on a HF diet than wild-type littersmates (33), supporting a role for IAP in body weight control. In our study, the LF group did not split into two subgroups regarding IAP activity, suggesting a nongenetic cause to the difference observed between DIO-R and DIO-P animals. When exposed to a HF diet, some rats seem able to respond with an increase in IAP activity. However, it is also possible that the decreased IAP activity precedes or follows inflammation, but likely contributes to the establishment or persistence of gastrointestinal inflammation and elevated plasma levels of LPS in obese animals.

In obese animals, an increase in MLC phosphorylation and increase in occludin in the cytoplasm of enterocytes was observed. Cytokines such as TNF-α and interferon-γ have been shown to increase myosin light chain kinase expression, leading to MLC phosphorylation and contraction of the cytoskeleton, which leads to disruption of tight junctions (40). Alteration in occludin distribution had been reported in ob/ob mice (9), and in vitro on epithelial cells stimulated with proinflammatory cytokines (8), occludin was chosen as a marker of tight junction disruption. The increase in gut permeability observed in DIO-P rats can be attributed to their damaged epithelial barrier, a direct consequence of local inflammation. Disruption in the intestinal epithelial barrier may have a deleterious effect on the regulation of food intake directly or indirectly by allowing translocation of potent pathogens such as LPS. Indeed, in humans, energy intake has been shown to be associated with LPS plasma levels (2). However, this study did not clarify whether LPS influences weight gain or if plasma levels are being modulated by the food ingested. Nevertheless, germ-free animals seem protected against high-fat feeding-induced obesity (5), and CD14 (coreceptor) for LPS null mice are resistant to the obesigenic effect of a HF diet (5), and CD14 is upregulated due to the increase in MLC phosphorylation in DIO-P rats (10), supporting a putative role of LPS in the development of obesity. There is evidence that SATs, which were a major component of the HF diet used in this study, can act as ligands at the TLR4 receptor (31, 36). It is possible that the increase in TLR4-MD2 complex that was detected was due to the increase in saturated fat from the HF diet or due to both saturated fats and LPS activating TLR4. However, fatty acids from micelles are mostly absorbed at the midjejunum level during digestion (23), and TLR4 activation was investigated in the ileum.

In conclusion, this study showed a strong link between gut inflammation and obesity, and the ensuing increase in plasma level of LPS seems to play an important role. Thus the sequence of events could be an increase in luminal LPS due to altered gut microbiota, a decrease in IAP activity, and an increase in TLR4 activation at the epithelium, leading to altered tight junction permeability and an increase in gut inflammation. An increase in gut permeability could increase passage of LPS from the lumen to the lamina propria, resulting in an increase in plasma levels of LPS, “metabolic endotoxia” (see Fig. 8). The data suggest that development of intestinal epithelial inflammation in response to high-fat feeding may impair food intake regulation and be a possible triggering mechanism in the appearance of hyperphagia and obesity.

REFERENCES


