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Metabolic endotoxemia with obesity: is it real and is it relevant?

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Abstract

Obesity is associated with metabolic derangements in multiple tissues, which contribute to the progression of insulin resistance and the metabolic syndrome. The underlying stimulus for these metabolic derangements in obesity are not fully elucidated, however recent evidence in rodents and humans suggests that systemic, low level elevations of gut derived endotoxin (lipopolysaccharide, LPS) may play an important role in obesity related, whole-body and tissue specific metabolic perturbations. LPS initiates a well-characterized signaling cascade that elicits many pro-and anti-inflammatory pathways when bound to its receptor, Toll-Like Receptor 4 (TLR4). Low-grade elevation in plasma LPS has been termed “metabolic endotoxemia” and this state is associated with a heightened pro-inflammatory and oxidant environment often observed in obesity. Given the role of inflammatory and oxidative stress in the etiology of obesity related cardio-metabolic disease risk, it has been suggested that metabolic endotoxemia may serve a key mediator of metabolic derangements observed in obesity. This review provides supporting evidence of mechanistic associations with cell and animal models, and provides complimentary evidence of the clinical relevance of metabolic endotoxemia in obesity as it relates to inflammation and metabolic derangements in humans. Discrepancies with endotoxin detection are considered, and an alternate method of reporting metabolic endotoxemia is recommended until a standardized measurement protocol is set forth.

Keywords

Obesity; metabolic endotoxemia; substrate metabolism; inflammation; endotoxin detection

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Conflicts of Interest

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1. Introduction

Endotoxins are large, heat stable lipopolysaccharides (LPS), which are the major glycolipid component of the outer membrane of gram-negative bacteria[1] that comprise approximately 70% of the total bacteria in the gut [2]. Endotoxin can enter the blood by either local or systemic infection by exogenous gram-negative bacteria, through paracellular absorption following bacterial cell lysis of endogenous gram-negative bacteria in the gut, and through transcellular (via chlyomicrons) transport of endogenous endotoxin following diurnal feeding patterns[3, 4]. LPS contains a pathogen-associated molecular pattern, Lipid A, which initiates a signaling cascade resulting in activation of various pro-inflammatory pathways and increases oxidative stress upon binding to its pattern recognition receptor, Toll-like receptor 4 (TLR4)[4–8]. TLR4 resides on the cell surface of monocytes, other immune cells, and various other cell types (e.g., skeletal muscle, adipose tissue, and liver)[9–11].

Bacterial infections are the leading cause of sepsis, of which gram-negative bacterial infections account for of the 45–60% of cases[12, 13]. In patients with sepsis, the concentration of circulating endotoxin is often elevated a hundred fold or higher compared to age-matched, healthy controls (e.g., 581±49 vs. 5.1±7.3 pg/mL, respectively)[14]. The elevation in endotoxin in bacterial infection (endotoxemia) results in mass overproduction of pro-inflammatory cytokines, which can lead to shock, cell damage, and potentially multiple organ failure[15]. Conversely, Cani et al.[4] described “metabolic endotoxemia” as a condition of chronically elevated plasma LPS at levels 10–50 times lower than during septic conditions. Metabolic endotoxemia was observed in genetically obese (ob/ob) mice consuming normal chow, and could be induced in lean mice (C57bl6/J) consuming an obesogenic diet[4, 16]. Diet induced elevations in endotoxin were related to increased fat deposition, heighten pro-inflammatory and oxidative pathways and insulin resistance[4, 16], and these perturbations could be partially or completely abrogated with antibiotic treatment[4, 16]. Thus, these findings were the first to indicate gut microbial endotoxin as a mediating source of a heightened pro-inflammatory milieu in obese rodent models[16].

Approximately 65% of US adults and > 100 billion people worldwide are overweight or obese[17, 18]. Accompanying obesity is a state of chronic low-grade inflammation, characterized by elevated systemic and local pro-inflammatory cytokines and acute phase proteins[19]. Heightened activation of the immune system in obesity plays a role in the development of the Type II diabetes (T2D), the metabolic syndrome, and cardiovascular disease[20, 21]. Importantly, cardiovascular disease and T2D are the 1st and 7th leading cause of death in the United States, respectively[22, 23].

Local adipose tissue inflammation and the secretion of a plethora of pro-inflammatory adipokines from visceral adipose tissue is indicated in the etiology of cardio-metabolic disease development during obesity[24]. However, the finding of Cani and colleagues, in association with recent evidence in humans, has lead to the hypothesis that metabolic endotoxemia may also participate in low-grade inflammation and the development of cardio-metabolic disease in obesity[25–27]. The present review will provide an overview of the evidence of the existence, consequences, and clinical relevance of metabolic endotoxemia in human obesity, and supports mechanistic associations with cell and rodent experiments. In

addition, issues of endotoxin detection as it relates to measuring and reporting metabolic endotoxemia concentrations in the literature are discussed.

2. Metabolic endotoxemia and obesity: Evidence in rodents

2.1. Metabolic endotoxemia and its involvement in obesity, inflammation, and insulin resistance

It is important to note that the influence of bacterial endotoxin on metabolism has been studied for nearly a century[28, 29]. Initial studies explored the effects of lethal doses of endotoxin on metabolism, while later studies evolved to explore similar metabolic outcomes with sub-lethal concentrations of endotoxin or during bacterial sepsis[30–33]. More recently, the influence of chronically elevated plasma LPS, at levels 10–50 times lower than during septic conditions, on metabolism has been characterized and termed, “metabolic endotoxemia”[4].

In a series of studies, Cani et al. showed that chronic, modest elevations (~ 1.5 fold) in endotoxin could be induced in lean mice (C57bl6/J) consuming a high-fat (72% of total calories)/high energy diet for four weeks or in genetically obese (ob/ob) mice consuming normal chow[4, 16]. In these studies, the diet induced elevations in endotoxin were related to increased fat deposition, systemic and tissue specific inflammation (e.g., liver, skeletal muscle, and adipose tissue) and insulin resistance[4, 16].

The role of endotoxin as a mediator of adipose tissue development, systemic and local inflammatory processes and metabolic derangements was confirmed through low dose LPS (300 μg/kg/day) injection in lean mice on a normal chow diet[4]. Injection of 300 μg/kg/day of LPS in lean mice elicited similar derangements of diet induced obesity, however these mice developed slightly less glucose intolerance compared to mice consuming an obesogenic diet. Furthermore, lean mutants lacking the crucial LPS co-receptor, cluster of differentiation (CD) 14, were resistant to high fat diet induced weight gain, tissue specific inflammation, hepatic lipid deposition, and insulin resistance, therefore indicating TLR4 activation via LPS as a mediating event in high fat diet induced inflammation and metabolic derangements[4].

Follow-up studies in lean mice (C57bl6/J) showed that consuming either high-carbohydrate (37% of total calories)/ high-energy or high-fat (72% of total calories)/high energy diets for four weeks lead to significant increases in circulating plasma endotoxin compared to mice consuming an isocaloric control diet for the same duration[34]. However, the increase in plasma endotoxin was significantly greater in the high-fat/ high-energy diet group compared to the high-carbohydrate/ high-energy group (~2.5 fold increase vs. ~1.5 fold increase, respectively) indicating that both dietary composition and energy intake influence the magnitude of elevation in circulating endotoxin.

2.1.2. The gut microbiota serves as the link between high fat feeding, endotoxin, and inflammation

Under normal physiological conditions, the gut microbiota promotes gut barrier function through a glucagon like peptide (GLP) 2 dependent mechanism[35]. However, a high fat diet can unfavorably alter the gut microbial composition, leading to increased intestinal

permeability, as evidenced by less abundant and disorganized tight junction proteins, zonulin and occludin in the colon[35]. The gut microbiota dependent mechanisms mediating increased intestinal permeability are not fully elucidated, however a reduction in *Bifidobacterium spp*. and overactivation of the endocannabinoid (eCB) system seem to play an important role[16, 35, 36]. Furthermore, LPS, which is elevated due to increased intestinal permeability, can increase intestinal permeability and the peripheral eCB tone, thus completing a damaging positive feedback pathway[36]. Administration of antibiotics or prebiotics to genetically (ob/ob) or diet induced obese mouse models leads to a reduction in intestinal permeability and circulating plasma endotoxin[16, 35, 36]. Notably, selective modulation of the gut microbiota with prebiotics has been shown to decrease the mRNA expression of the eCB receptor, CB_1 in the colon; decrease the eCB agonists, anandamine and 2-aracidonoylglycerol; decrease the expression of eCB agonist inhibitor, fatty acid amide hydrolase; and increase the endogenous production of the intestinotrophic, GLP2 in mice administered a high fat diet[35, 36]. Together the improvements in gut barrier function reduce circulating LPS, inflammation, and metabolic derangements; thus indicating that changes in the gut microbiota mediate metabolic endotoxemia and systemic and local inflammation.

2.2. Metabolic endotoxemia and altered adipose, liver and skeletal muscle metabolism

Endotoxin from LPS can initiate systemic and local inflammation and also result in reactive oxygen species (ROS) production upon binding with TLR4 and subsequent activation of NFκB[6–8]. TLR4 is abundant on immune cells, liver, adipose tissue, and skeletal muscle[9–11]. Collectively, these tissues play an important role in the regulation of glucose and lipid homeostasis, and it has been demonstrated that pro-inflammatory cytokines and ROS production interfere with normal metabolism in these tissues[37–40]. For instance, Cani et al.[35] have reported increased expression of pro-inflammatory (e.g., PAI-1 TNFa, IL6, IL-1), oxidative stress (NADPHox, iNOS) and macrophage infiltration markers (CD86) in liver tissue of genetically obese mice with metabolic endotoxemia. In addition, increased expression of pro-inflammatory markers have been observed in visceral and subcutaneous adipose tissue of lean mice after chronic low dose LPS injection, with concomitant increases in weight gain, visceral and subcutaneous adipose tissue size, and increased liver weight[4]. Interestingly, CD14 knockout mice do not experience these inflammatory changes with LPS injection; nor do they develop changes in body weight, adipose tissue size, and increased liver weight. The mechanisms of altered lipid metabolism with LPS stimulation are unclear. However, increased adipose tissue size following LPS exposure appears to occur, at least in part, through a decrease in adipogenesis[36].

Although, some have reported increased pro-inflammatory expression in skeletal muscle following chronic low dose LPS injection (300 μg/kg/day)[4], others report altered fasting skeletal substrate metabolism in the absence of pro-inflammatory expression in lean mice following a single low dose LPS injection (0.025 μg)[41]. Specifically, Frisard et al.[41] demonstrated a switch in basal (fasting) substrate preference in rodent skeletal muscle cultures to increased glucose oxidation and decreased fatty acid oxidation after a single 2-hour LPS treatment (500 ng/mL of *Escherichia coli* 0111:B4) (Figure 1A–B). This switch in substrate preference was accompanied by an increase in lactate production and an increase in neutral lipid synthesis (Figure 1C–D). Interestingly, low dose LPS (50 pg/mL) elicited the

same effects as 500 ng/mL after a single 2-hour exposure. The use of TLR4 mutant mice confirmed a TLR4 dependent pathway for changes in basal substrate metabolism in skeletal muscle since TLR4 mutant mice were resistant to reductions in basal fatty acid oxidation and increases in glucose oxidation after a single low dose LPS injection (0.025 μg/mouse). Treatment with parthenolide, an inhibitor of IκB Kinase (IKK), in C2C12 (mouse myotubes) cells resulted in complete abrogation of substrate metabolism changes with low dose LPS treatment. This data suggests that the metabolic perturbations in skeletal muscle are due, at least in part, to NFκB translocation via LPS-TLR4 activation. Follow-up studies in C2C12 cells demonstrated decrements in mitochondrial respiration following short-term (2 hour), low dose (50 pg/mL) LPS treatment[42]. In addition, co-treatment with LPS and the antioxidant, n-acetyl-cysteine (NAC), prevented changes in cellular mitochondrial respiration and changes in basal substrate metabolism previously reported (Figure 2A–D). Taken together, short term LPS exposure induces changes in basal skeletal muscle metabolism and mitochondrial oxygen consumption that seem to depend on TLR4 activation, NFκB translocation and ROS production. Indeed, LPS has been shown to increase ROS production via direct TLR4 activation and NFκB translocation in other cell types[43]. Furthermore, ROS has been demonstrated to activate NFκB translocation, therefore completing a noxious positive feedback pathway that is likely to participate in aberrant skeletal muscle metabolism following LPS exposure[44].

The skeletal muscle metabolic changes in the absence of pro-inflammatory activation in our experiments may be due to the fact that our “low dose” LPS treatment is ~300x lower than what has been previously reported as “low dose” LPS by Cani et al.[4]. Standardizing the terminology of LPS dosage is needed to clarify mechanisms responsible for metabolic changes following LPS exposure. It appears that ‘very’ low concentrations of LPS are below a threshold needed to trigger inflammatory pathways, but not below a threshold to alter skeletal muscle metabolism. In accordance with this hypothesis, perturbations in metabolism have been observed in human leukocytes following “low dose” LPS exposure in the absence of a pro-inflammatory response, while higher doses elicit metabolic alterations in tandem with an increased pro-inflammatory signature[45]. Future studies are needed to determine if a similar phenomenon is apparent in other cell types.

3. Metabolic endotoxemia and obesity: Evidence in humans

3.1. Increased TLR4 tissue expression, elevated circulating endotoxin, and altered gut microbiota in obese vs. lean individuals

Obesity is characterized as a chronic state of low-grade inflammation[46, 47]. Complimentary cross-sectional evidence indicates that the LPS-TLR4 pathway is responsible, at least in part, for the heightened pro-inflammatory milieu in human obesity. For example, Creely et al.[48] reported that TLR4 mRNA expression and protein content is elevated in adipose tissue from obese and T2D compared to lean donors. In addition, similar findings have been reported, in tandem with increased TLR4 signaling, in skeletal muscle from obese and T2D compared to normal weight donors[41, 49]. Furthermore, cross-sectional analyses have shown an elevation in circulating endotoxin in obese individuals compared to their lean counterparts [25, 50]. To tie things together, Ley et al.[51] were the first to describe compositional differences in the gut

microbiota of obese compared to lean individuals. At the phylum level, it was found that obese individuals had a greater firmicutes/bacteroidetes ratio than aged matched controls and this difference was reversed with either carbohydrate-restricted or fat-restricted diets. Notably, and possibility due to differences in measurement techniques, not all have found these same differences in the gut microbiota of obese individuals, with some finding the opposite[52, 53]. More recently, there has been evidence of reduced bacterial diversity and specific genus and species differences in the gut microbiota of obese individuals[5455]. Specifically, representatives of the genus *bifidobacterium* are reduced in the feces of obese subjects[52, 56, 57]. Interestingly, the abundance of *bifidobacterium* is associated with gut barrier function and reduced intestinal endotoxin levels in rodents[58, 59]. As mentioned previously, the gut microbiota serves as the link between high fat feeding, endotoxin translocation, and systemic inflammation in rodent models. Thus, it is possible that this relationship also exist in humans; however the cross-sectional nature of these studies limits this interpretation. Therefore, prospective and experimental studies have been designed and executed to further elucidate this relationship in humans, as summarized below (See Sections 3.2–3.5).

3.2. High fat meals, short-term high fat diets, and metabolic endotoxemia

A high fat meal induces a modest elevation in circulating endotoxin in healthy, non-obese, obese and T2D individuals compared to fasting concentrations[60–64]. Postprandial elevations in circulating endotoxin peak between 1 and 5 hours, are transiently elevated (~ 1–2 hours), and appear to be more robust in metabolically impaired individuals[60–64]. In line with rodent studies, the elevation in postprandial endotoxin occurs in tandem with heightened pro-inflammatory pathway activation and ROS generation. For example, increased circulating sCD14 and IL-6; increased NFκB DNA binding; increased TLR4 protein expression; increased SOCS3, TNFα, and IL-1B mRNA expression in mononuclear cells; and increased protein expression of NADPH oxidase subunit $p47^{phos}$ in mononuclear cells has been observed in the postprandial period alongside elevations in circulating endotoxin[61, 63, 65]. In lean rodents, 4 weeks of an obesogenic diet leads to elevated fasting plasma endotoxin and a disruption in the natural diurnal rhythm of circulating plasma endotoxin[4]. Our group very recently corroborated these findings in lean, healthy humans fed a short-term (5 days) isocaloric high fat diet [55% fat, (25% of total energy as saturated fat), 30% carbohydrate, and 15% protein][64]. Specifically, this high fat diet significantly increased fasting serum endotoxin (Figure 3A) and disrupted the normal fasted-to-fed serum endotoxin production following a high fat meal (63% fat) (Figure 3B). Interestingly, the high fat diet completely abrogated postprandial elevations in glucose oxidation, and phosphorylation status of p38 protein following a high fat meal (Figure 3C–F), which occurred in the absence of changes in whole body insulin sensitivity. Ongoing, larger studies are exploring the mechanisms behind these changes (NCT02328235- Hulver, P.I.) On the other hand, Hulson et al.[66] report that an acute (7 days) hypercaloric (~ 50% above energy needs) high fat diet (65% fat) impairs insulin sensitivity in healthy, non-obese individuals and leads to significant weight gain; whereas normal insulin sensitivity and body weight is maintained in those consuming a probiotic mixture (*Lactobacillus casei* Shirota) for 4-weeks. Although these researchers did not measure circulating endotoxin, these findings suggest that maintenance of a favorable gut microbiota composition via probiotic supplementation likely prevent changes in insulin

sensitivity. However, whether the maintenance of a healthy microbiota during high fat feeding prevented elevations in circulating endotoxin, and thus preserved whole body glucose metabolism merits future study.

3.3. Long-term high fat diets and metabolic endotoxemia

The reports of a relationship between longer term high fat feeding (4–8 weeks) and elevated circulating endotoxin are inconsistent. For instance, Pendyala and colleagues[67] show that plasma endotoxin is significantly elevated (71% vs. baseline), independent of increases in circulating pro-inflammatory cytokines, after 1 month of isocaloric, high fat (40% fat of total energy) feeding in middle aged (55–66 years old), healthy adults. Conversely, 8 weeks of high fat (45% fat of total energy) overfeeding (+ 760 kcals/day) did not influence fasting plasma endotoxin in healthy, non-obese males [68]. Furthermore, we recently corroborated the lack of change in circulating serum endotoxin, whole body insulin sensitivity, and skeletal muscle substrate metabolism after 4 weeks of high fat (55% fat of total energy) overfeeding (+1000 kcal/day) in healthy, young non-obese males (*unpublished findings, in review*). Obviously, the relationship between high fat feeding and metabolic endotoxemia is more complex in humans compared to rodents and appears to be influenced by the time course of feeding, the macronutrient (and possibly energy) composition, and the age of participants. Notably, each of the above mentioned studies measured endotoxin activity with different assays, thus likely contributing to these variable findings. Future studies are needed to determine whether short and long-term high fat diets exacerbate existing elevations in circulating endotoxin in obese and T2D individuals.

3.4. Observational and experimental clinical implications of metabolic endotoxemia in obesity and cardio-metabolic disease

Large prospective population based studies highlight the clinical relevance of elevated circulating endotoxin and proteins of the LPS-TLR4 pathway as they relate to cardio-metabolic disease development. For example, elevated baseline fasting serum endotoxin concentrations predicted coronary heart disease events at follow up (10 years) in middle aged, otherwise healthy individuals[25]. In addition, the risk of incident diabetes is increased at follow up (10 years) in healthy adults aged 25–75 with elevated serum endotoxin[69]. Furthermore, the risk for developing metabolic syndrome and most of its components (including central adiposity) is increased in middle aged and older healthy Chinese adults with elevated fasting plasma LPS binding protein (LBP) at baseline [70]. Indeed, the role of endotoxin as a key mediator in the development cardio-metabolic disturbances cannot be gleaned from longitudinal observation studies. Fortunately, many studies have induced experimental endotoxemia via intravenous injection of endotoxin to address this issue.

Administration of lower to moderate doses (0.2–2 ng/kg body weight) of *E coli* LPS produce pro-inflammatory and metabolic disturbances that more closely resemble chronic diseases than do higher (3–5 ng/kg body weight) doses[71]. For instance, low dose (0.6 ng/kg body weight) intravenous LPS administration induces rapid, transient increases in plasma IL-6 (25-fold) and TNFα (100-fold), followed by modest induction of pro-inflammatory mRNA cytokine expression in adipose tissue (i.e., IL-6, TNFα, MCP-1, SOCS1 and SOCS3)[72]. In addition, whole body insulin sensitivity (Si) and HOMA- IR are significantly impaired post

24 hours LPS injection, independently of altered β-cell function to levels commensurate of metabolic syndrome and diabetes[73, 74]. Others have observed decreased basal and insulin stimulated skeletal muscle glucose uptake, in the absence of increased circulating pro-inflammatory cytokines, while continuously infusing low dose LPS (0.025 ng/kg/hour) over 360 minutes into the femoral artery[75]. Skeletal muscle biopsies did not reveal that LPS impaired insulin signaling compared to placebo in this study. A single intravenous dose (2 ng/kg of body weight) of LPS increases NEFA[76, 77], indicative of increased adipose tissue lipolysis[76, 77]. According to the reverse Randle cycle, it is possible that the LPS induced rise in circulating FFA could reduce glucose uptake and oxidation without impairing insulin signaling[78, 79]. These observations are in contrast to a switch to preferential glucose oxidation over fatty acid oxidation in the fasting period in rodent skeletal muscle following acute LPS exposure[41, 42]. However it is important to note that these studies use different LPS dosing paradigms, therefore the continuous infusion of LPS over 360 minutes in the former study[75] may alter skeletal muscle metabolism differently than a single LPS injection or short LPS exposure[41, 42]. Indeed, future studies are needed to elucidate these differences.

Single low-dose experimental endotoxemia models provide causal evidence of LPS induced metabolic disturbances that closely resemble those observed in obesity and the metabolic syndrome. However, this model has its limitations, as it does not accurately reflect low-grade chronically elevated endotoxin observed after high fat feeding and metabolic disease. In rodents, subcutaneously implanted mini-osmotic infusion pumps have been used to continuously deliver LPS at low dose (300 μg/kg/day) for 4-weeks, which lead to similar circulating endotoxin levels and metabolic derangements as observed following high fat feeding for the same duration[4]. Continuous infusion regimens have been performed in humans with other drugs, such as chemotherapy[80]. To our knowledge, continuous delivery of LPS at low doses has not been performed in human subjects, but warrants future study to more accurately model metabolic endotoxemia. The importance of developing these studies is reflected in the diminished the pro- and anti-inflammatory responses to LPS on day 5 compared to day 1following once daily intravenous injections of 2ng/kg/day of *E. coli.* LPS for 5 consecutive days[81]. Whether a resistance to inflammatory and metabolic changes develops to continuous low dose delivery of LPS is unknown.

3.5. Weight loss, gut microbiota changes, and reductions in metabolic endotoxemia

As reviewed above, the prevailing hypothesis is that high energy/fat intake leads to gut microbiota dysbiosis and leads to elevations in circulating endotoxin in humans through increased paracellular (increased intestinal permeability) and transcellular transport (chylomicron-LPS co-transport) [3, 4, 35]. Therefore, reducing caloric intake and/or modulating the gut microbiota (e.g., pre-, pro-, or synbiotics) may reduce circulating endotoxin and metabolic perturbations. Some studies show that bariatric surgery leads to altered gut microbiota[82], reductions in circulating endotoxin[27, 83], reductions in pro-inflammatory pathways[27, 82, 83], and improvements in glucose homeostasis[27, 82, 83]. Monte et al.[27] showed that, in addition to reductions in circulating endotoxin, TLR4, TLR2, and CD14 mRNA expression, and NFκB binding to DNA in mononuclear cells were all significantly reduced following Roux-en-Y gastric bypass surgery (RYGB) surgery. Furthermore, by measuring bacterial DNA (broad range PCR and of prokaryote 16sRNA) in

plasma, Ortiz et al.[83] only showed reductions in pro-inflammatory pathways and insulin resistance following surgical (RYGB and sleeve gastrectomy) weight loss in subjects who had concomitant reductions in circulating bacterial DNA. However, these results should be interpreted with caution since only a small number of subjects had increases or no change in circulating bacterial DNA content following weight loss. Furthermore, in these studies it is unclear whether reduced caloric intake, changes in the gut microbiota, or a combination of both, are responsible for the reductions in circulation endotoxin following weight loss.

Diet-induced weight loss, with or without gut modulation therapy, has been shown to reduce circulating endotoxin, reduce systemic inflammation, and improve glucose homeostasis in obese individuals and T2D[84–87]. In one study, the changes in the proportions of firmicutes, bacilli, actinobacteria, *Bifidobacterium* and *Faecalibacterium praausnitzii* were negatively corrected to the change in circulating endotoxin in obese women following 8 weeks of prebiotic therapy (inulin-type fructans; 16g/day). In addition, Zhao et al.[88] recently reported reductions in intestinal permeability, several genera of endotoxin producing gut bacteria, systemic pro-inflammatory tone, and improved insulin sensitivity following a 9-week multiple faceted weight loss intervention that included prebiotic consumption. Following the intervention, the proportion of *Citrobacter* (genera of bacteria containing opportunistic pathogens and LPS containing microbiota) was positively correlated to body mass, body mass index, and IL1-β after adjusting for gender, weight loss, and age. This evidence suggests that the gut microbiota may serve as the link between high fat intake, endotoxin translocation, and systemic inflammation in humans. To support this evidence further, prebiotic (xylo-oligosaccharide+ inulin; 1g and 3g/ day, respectively) treatment in young, healthy individuals consuming a moderately high fat diet was able to reduce circulating endotoxin concentrations, in the absence of weight loss[89]. In addition, the prebiotic treatment attenuated increases in IL1-β (pro-inflammatory) gene expression and lessened the decrease in IL-13 (anti-inflammatory) gene expression in whole blood stimulated with LPS during following the intervention. Taken together, since weight loss is known to alter the gut microbiota, and gut modulation therapy has been shown to reduce circulating endotoxin and its consequences without weight loss, it appears the gut microbiota alterations mediate endotoxin translocation and systemic inflammation in humans.

4. Endotoxin Detection: Limitations with measurement in blood

Multiple methods of endotoxin detection are available and the strengths and limitations of these methods have been reviewed elsewhere [1, 15, 90, 91]. The FDA accepts the use of a Limulus Amoebocyte Lysate (LAL) test for detection of endotoxins. The LAL test is based on a conserved coagulation cascade that is initiated in amoebocytes of the Horseshoe Crab when LPS from gram-negative bacteria is introduced to the host[15]. The cascade involves three serine protease zymogens and one clottable protein zymogen. The first step of the clotting cascade, Factor C, is activated in the presence of LPS, leading to a series of zymogen activations leading to a coagulin gel clot. The degree of activation of this cascade is related to endotoxin activity[1, 15]. Endotoxin concentrations can be reported in a weight by volume measure (e.g., pg/mL) or by endotoxin units (EU) per unit volume (e.g., EU/mL). EU is a measure of endotoxin activity and is preferred to the weight per volume

measurement since LPS from different gram-negative bacteria have different potencies that depend on multiple factors[92].

Despite its wide spread use, the LAL has inherent drawbacks in seasonal preparation and contamination with 1–3 β-D glucan leading to decreased and sensitivity and specificity, respectively[15]. In addition, the detection of endotoxin in the blood is widely regarded as difficult and susceptible to inaccuracies, with many factors in plasma (bile salts, proteins, co-factors, and lipoproteins) inhibiting LPS or rendering it non-detectable with the LAL test[1, 15]. In addition, anticoagulants used in plasma collection, such as EDTA and heparin have been shown to interfere with the LAL reaction[93, 94]. Furthermore, both false positive reactions and enhancement of endotoxin activity have been noted with the LAL in plasma samples[1]. Some, but not all, have also reported endotoxin detection issues in serum[95–97]. The generation of a recombinant Factor C allowed for the development of a single step activation endotoxin detection assay, thus, reducing inference of blood factors on steps beyond Factor C[98]. However, the factors in blood that interferes with LPS detection are not avoided with this assay. Given the difficulties with blood samples, heat inactivation and/or dilution of the sample are necessary to reduce interference[99]. In addition, chemical treatments are used when inactivation with heat is not effective[100]. Non-standardized treatment of blood samples has lead to wildly variable reports of endotoxin concentration in the literature (see below). In addition, the physiological relevance of measuring endotoxin in the blood after inactivation procedures has been questioned, since the pre-inactivated blood sample may more closely represents *in vivo* conditions of endotoxin exposure[101]. Therefore, it is possible that inactivation procedures to lead spurious endotoxin values not seen by LPS-sensing cells and tissues.

4.1. A range of metabolic endotoxemia: Does it exist and does it matter?

The range of endotoxin concentrations in healthy and obese/T2D individuals is wildly variable in the literature[26, 34, 50, 61, 89, 102, 103]. For example, Nádházi and colleagues[102] report a plasma endotoxin concentration between the range 0.01–1 EU/mL, with an average value of 0.128±0.215, in 116 healthy donors. Conversely, Lassenius et al.[26] report an average concentration 600x these values (60 ±9 EU/mL) in 345 healthy individuals. To complicate matters more, others have reported endotoxin concentrations of septic individuals below 1 EU/mL[104]. Furthermore, some groups report endotoxin concentrations in pg/mL, with absolute values of endotoxin differing greatly between studies[25, 60, 83]. Therefore, distinguishing an absolute range of metabolic endotoxemia using the description of metabolic endotoxemia as a condition of chronically elevated plasma LPS at levels 10–50 times lower than during septic conditions is near impossible when comparing between endotoxin concentrations of healthy, obese/T2D, and septic individuals across studies. It is also near impossible to determine an endotoxin concentration at which cardio-metabolic and pro-inflammatory symptoms ensue during metabolic endotoxemia. Conversely, what is consistent in the human studies reviewed herein is that the endotoxin concentration changes between lean and obese individuals, and decreases in circulating endotoxin concentration following weight loss and/or gut modulation therapy[27, 83, 84, 86, 87, 89, 105], have been between 0.5–2 fold[25, 26, 50, 6127, 83, 84, 86, 87, 89, 105]. Furthermore, the 0.5–2.0-fold change in endotoxin has been accompanied by concomitant changes in metabolism, with or without

changes in immune system activation in these studies. Therefore, a 0.5–2.0-fold increase in circulating endotoxin may be a better indicator of metabolic endotoxemia than the traditional definition until standardization methods for endotoxin detection are set forth.

5. Conclusions

Obesity is at epidemic proportions in the United States and worldwide and excess adiposity is related to progression of many cardio-metabolic diseases. The mechanism(s) underpinning the relationship between excess adiposity and cardio-metabolic risk are not fully elucidated, however oxidative stress and inflammation play an important role. Long withstanding evidence has attributed the pro- inflammatory and oxidant milieu in obesity to local adipose issue inflammation and the secretion of activating adipokines. However, strong, evidence in rodents has defined a novel pathway linking gut microbiota derived endotoxin to elevated pro-inflammatory and pro-oxidant activation and subsequent metabolic derangements in overfeeding and obese models. Supporting evidence suggests that elevated systemic LPS, below levels that activate the immune system, alter substrate metabolism in skeletal muscle--a predominate site of substrate disposal whose dysregulation is associated with multiple metabolic disorders. This cumulative evidence in rodents has led to efforts to translate these basic science findings in rodents to human participants.

Not surprisingly, evidence from human studies have less clearly defined the association between metabolic endotoxemia and obesity compared to rodents studies. However, complimentary findings that span cross-sectional, prospective, longitudinal and experimental studies highlight the clinical relevance of metabolic endotoxemia in obesity as it relates to inflammation and cardio-metabolic derangements. The specific bacterial communities responsible for the association between the gut microbiota and metabolic endotoxemia remain controversial.

An important issue that merits future clarification and standardization is endotoxin detection in blood samples. Due to assay and measuring discrepancies, the report of endotoxin values is highly variable and inconsistent between studies. Conversely, relative fold changes in circulating endotoxin are surprisingly consistent between studies, regardless of the absolute values and units provided. Therefore we postulate that 0.5–2.0-fold increase in circulating endotoxin may be a better indicator of metabolic endotoxemia than what has been traditionally defined. Taken together, although difficult to define, it appears that metabolic endotoxemia is both real and relevant in human obesity. Metabolic endotoxemia is related to systemic and local inflammation and therefore may contribute, at least in part to cardio-metabolic disease risk associated with obesity. What remains to be further explored are the mechanism(s) and impact of the pro-inflammatory- independent actions of low levels of LPS on altered substrate metabolism in human obesity and metabolic diseases.

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Abbreviations

LPS	lipopolysaccharide
TLR4	Toll-Like Receptor 4
T2D	Type II Diabetes
GLP2	Glucagon Like Peptide 2
eCB	endocannabinoid
ROS	reactive oxygen species
CD	cluster of differentiation
IKK	IκB kinase
NAC	n-acetyl cysteine
NFκB	nuclear factor kappa-light-chain-enhancer of activated B cells
LBP	LPS binding protein
Si	insulin sensitivity
HOMA-IR	homeostatic model assessment of insulin resistance
RYGB	Roux-en-Y gastric bypass surgery
LAL	Limulus amebocyte lysate
EU	endotoxin unit

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Highlights

- Low-grade elevation in plasma lipopolysaccharide (LPS) has been termed “metabolic endotoxemia” and this state is associated with a heightened pro-inflammatory and oxidant environment often observed in obesity
- This review provides supporting evidence of mechanistic associations with cell and animal models, and provides complimentary evidence of the clinical relevance of metabolic endotoxemia in obesity as it relates to inflammation and metabolic derangements in humans.
- Discrepancies with endotoxin detection are considered, and an alternate method of reporting metabolic endotoxemia is recommended until a standardized measurement protocol is set forth.

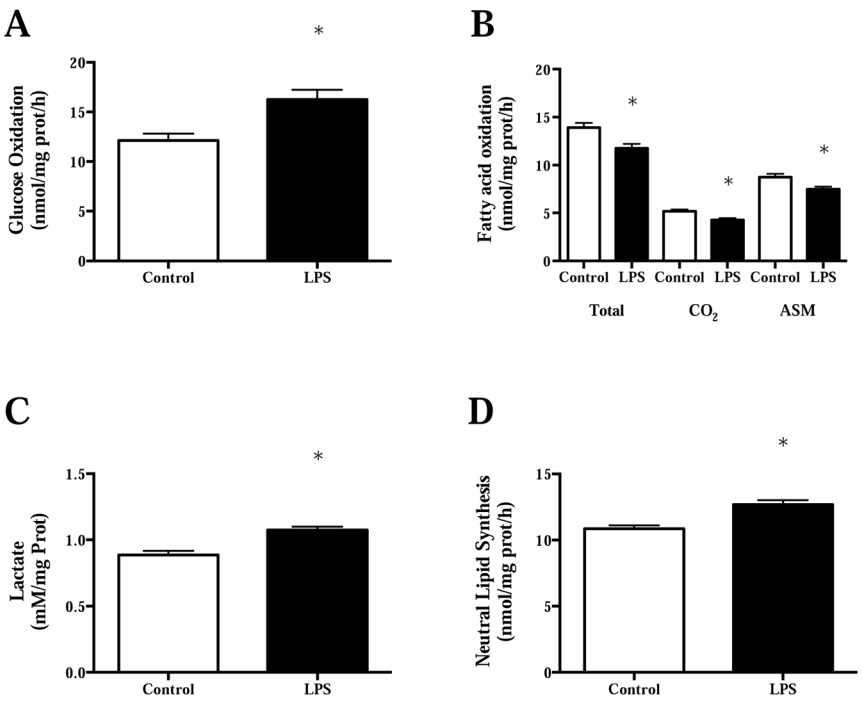

Figure 1.

A) Glucose oxidation, B) fatty acid oxidation (Total= total palmitate oxidation (CO_2 + ASM); CO_2=complete palmitate oxidation; ASM= acid soluble metabolites and represents incomplete palmiate oxidation), C) lactate production, and D) neutral lipid synthesis in C2C12 cells following 2 hour treatment with lipopolysaccharide (500 ng/mL). *P<0.05 compared to control. Data are presented as mean± SE. Figure redrawn from Frisard et al.[41].

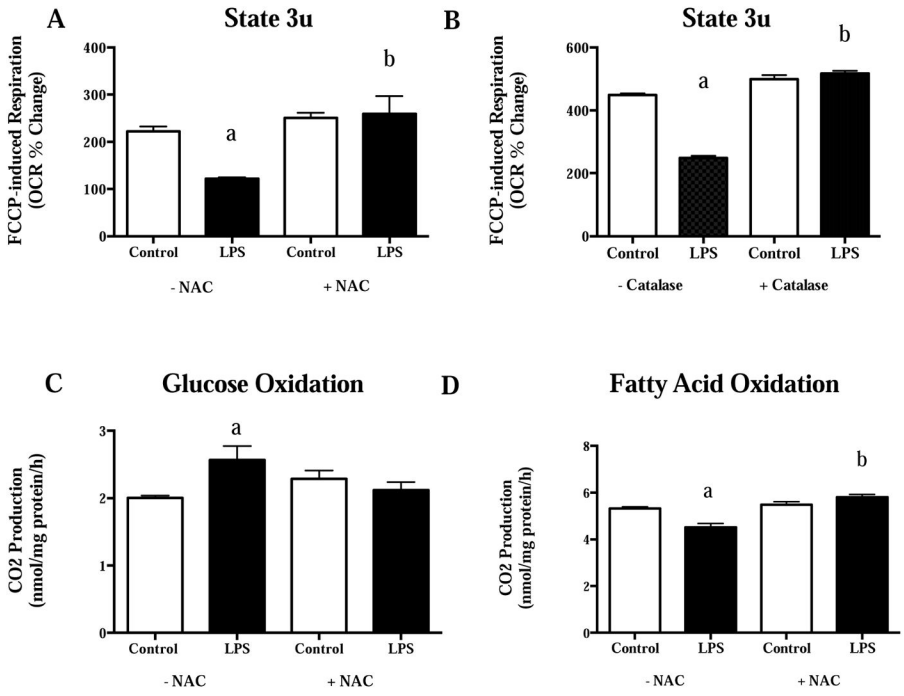

Figure 2.

A) FCCP-stimulated maximal respiration with and without 20 mmol/L NAC, B) FCCP-stimulated maximal respiration with and without 25 U/mL of catalase, C) glucose oxidation with and without 20 mmol/L NAC, and D) fatty acid oxidation with and without 20 mmol/L NAC in C2C12 cells following 2-hour LPS treatment (50 pg/mL). a, significantly different from control without NAC/Catalase co-treatment, $P<0.05$. b, significantly different from LPS treatment without NAC/Catalase co-treatment, $P<0.05$. Data are presented as mean± SE. FCCP= Carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone; NAC=N-acetyl cysteine. Figure redrawn from Frisard et al.[42].

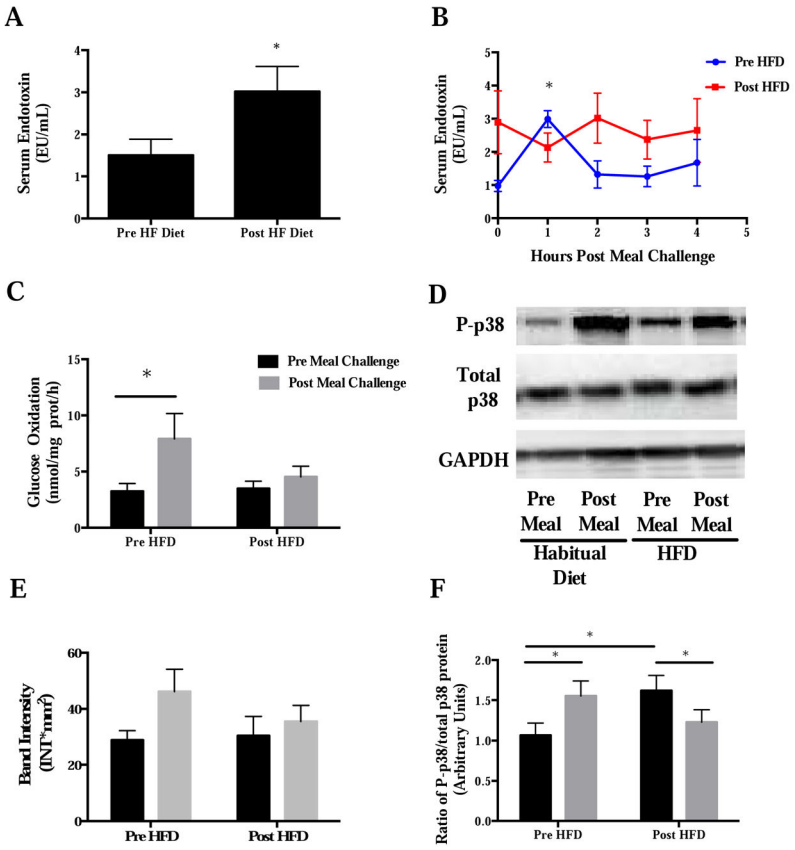

Figure 3.

A) Fasting serum endotoxin, B) postprandial serum endotoxin, C) fasting and postprandial (4 hour post high fat [63%] meal) glucose oxidation, DE) fasting and postprandial p38 MAPK total and phosphorylated protein, and F) fasting and postprandial phospho- to total-p-38 ratio before and after a 5 day, eucaloric high fat (55%) diet in healthy, non-obese males (n=6). *P<0.05. Data are presented as mean± SE. Figure redrawn from Anderson et al.[64].