Intestinal Lipid Handling
Evidence and Implication of Insulin Signaling Abnormalities in Human Obese Subjects

Alain Veilleux,* Émilie Grenier,* Picard Marceau, André C. Carpentier, Denis Richard, Emile Levy

Objective—Animal models have evidenced the role of intestinal triglyceride-rich lipoprotein overproduction in dyslipidemia. However, few studies have confronted this issue in humans and disclosed the intrinsic mechanisms. This work aimed to establish whether intestinal insulin resistance modifies lipid and lipoprotein homeostasis in the intestine of obese subjects.

Approach and Results—Duodenal specimens obtained from 20 obese subjects undergoing bariatric surgery were paired for age, sex, and body mass index with or without insulin resistance, as defined by the homeostasis model assessment of insulin resistance. Insulin signaling, biomarkers of inflammation and oxidative stress, and lipoprotein assembly were assessed. The intestine of insulin-resistant subjects showed defects in insulin signaling as demonstrated by reduced protein kinase B phosphorylation and increased p38 mitogen-activated protein kinase phosphorylation, likely as the result of high oxidative stress (evidenced by malondialdehyde and conjugated dienes) and inflammation (highlighted by nuclear factor-κB, tumor necrosis factor-α, interleukin-6, intercellular adhesion molecule-1, and cyclooxygenase-2). Enhanced de novo lipogenesis rate and apolipoprotein B-48 biogenesis along with exaggerated triglyceride-rich lipoprotein production were observed, concomitantly with the high expression levels of liver and intestinal fatty acid–binding proteins and microsomal transfer protein. The presence of an aberrant intracellular cholesterol transport/metabolism was also suggested by the reduced expression of ATP-binding cassette A1 transporter and proprotein convertase subtilisin/kexin type 9.

Conclusions—According to the present data, the small intestine may be classified as an insulin-sensitive tissue. Dysregulation of intestinal insulin signaling, possibly triggered by oxidative stress and inflammation, was associated with exaggerated lipogenesis and lipoprotein synthesis, which may represent a key mechanism for atherogenic dyslipidemia in patients with metabolic syndrome. (Arterioscler Thromb Vasc Biol. 2014;34:644-653.)

Key Words: diabetes mellitus, type 2 ■ dyslipidemias ■ inflammation ■ intestines ■ oxidative stress

Insulin resistance (IR) is the central feature of type 2 diabetes mellitus (T2D) and represents a major complication, commonly associated with atherogenic dyslipidemia. The latter is characterized by hypertriglyceridemia, elevated plasma very-low-density lipoprotein, reduced high-density lipoprotein (HDL), and presence of small, dense low-density lipoprotein (LDL). This atherogenic dyslipidemia is increasingly recognized as a postprandial phenomenon, because postprandial triglyceride-rich lipoproteins (TRLs) and chylomicron remnants have been implicated as significant risk factors for atherosclerosis. Exaggerated hepatic very-low-density lipoprotein production and impaired plasma triglyceride clearance are thought to play a key role in this disorder. However, mounting evidence underlines the active implication of the small intestine in postprandial lipid alterations.

It currently becomes clear that the small intestine is far from being a simple absorptive organ as it was previously thought. Indeed, recent studies have demonstrated that the small intestine regulates lipid metabolism in fed and fasting states and may, therefore, be central in lipid homeostasis in both normal physiology and pathophysiological conditions. Lipid synthesis as well as lipoprotein assembly and secretion occur in this tissue, and these metabolic pathways are sensitive to several hormones such as insulin. Maintaining normal lipid homeostasis seems to require an interaction between intestinal and hepatic metabolism to adequately manage dietary intake. Otherwise, an imbalance in the lipid metabolism may lead to dyslipidemia and to increased risks of cardiovascular diseases (CVDs).

Studies primarily on animal models (fructose-fed hamster; Psammomys obesus, JCR:LA-cp rat) have pointed out the...
dominant role of hepatic and intestinal TRL overproduction in dyslipidemia in both fasting and fed states. For example, dyslipidemia in insulin-resistant *Psammomys obesus* is associated with hepatic overproduction of triglyceride-rich very-low-density lipoprotein and with intestinal overproduction of chylomicrons. Insulin-resistant animals were also characterized by a dysfunctional intestinal and hepatic cholesterol homeostasis. Only few studies have confronted the relevant issue of these important findings to humans as did the pioneering work of Lewis’ group, using stable isotope tracer infusion. They have shown an association between the increase in apolipoprotein (Apo) B-48–containing lipoproteins and IR in humans. Nevertheless, the link between this exaggerated assembly and secretion of TLR and in situ IR in the gut is not yet fully understood.

Obesity has been proposed to predate and promote IR molecular defect(s) although this assumption has seldom been proven in human and was not addressed in the small intestine. Evidently, caution should be exercised when extrapolating results from experimental animal models because of differences among species. This is reinforced in view of the distinct intestinal response to insulin sensitization in humans, which could differ from that in animals. Therefore, an important question to address is whether the small intestine of obese individuals with systemic IR is endowed with impaired insulin responsiveness/signaling. Moreover, it is crucial to determine whether the mechanisms include oxidative stress and inflammation. Finally, efforts are necessary to explore whether these pathophysiological conditions trigger Apo B-48–containing lipoprotein overproduction in the small intestine of obese subjects.

The opportunity to obtain intestinal specimens from insulin-sensitive and insulin-resistant obese subjects allowed us to test the hypothesis that subjects with systemic IR display poor intestinal insulin sensitivity attributable to cellular and molecular defects in insulin signaling cascade, which are promoted by local oxidative stress and inflammation. As a result, abnormally high lipogenesis and lipoprotein production develops in the enterocyte.

### Materials and Methods

Materials and Methods are available in the online-only Supplement.

### Subjects and Tissue Sampling

Twenty subjects (10 women and 10 men) undergoing biliopancreatic diversion were assigned to 2 groups matched for age, sex, and body mass index (BMI), but with either relatively low (<3) or high (>7) IR according to their homeostasis model assessment of insulin resistance (HOMA-IR). Overnight fasting blood samples were drawn on the morning before surgery to assess lipid profile and glucose homeostasis. Duodenum specimens were obtained during the surgery. The intestinal samples were immediately transferred to the laboratory for intestinal organ culture to perform functional studies (eg, de novo lipogenesis, Apo B-48 biogenesis, and chylomicron assembly and secretion). The remaining tissue was immediately frozen in liquid nitrogen and stored at −80°C for subsequent oxidative stress and inflammation analyses.

### Results

#### Anthropometric and Metabolic Characteristics

As per our study design, all patients were obese and candidates for bariatric surgery, which allowed us to obtain the specimens of proximal intestine. Baseline characteristics of the subjects are listed in Table 1. Overall, the mean age was 42.6 years and the cohort had 50% men. The mean BMI was 53.8 kg/m² with a large range (42.6–65.2 kg/m²). The subjects were divided into 2 paired groups according to their HOMA-IR index. Although there were no differences in age, sex, and BMI between insulin-sensitive and insulin-resistant groups, an increase was evidenced in fasting insulin levels and HOMA-IR index, as well as in glycohemoglobin values in the insulin-resistant group. Table 2 shows the detailed lipid profile of insulin-sensitive and insulin-resistant subjects. Insulin-resistant subjects present higher triglyceride levels and lower HDL-cholesterol concentrations as compared with insulin-sensitive subjects. In view of their low HDL-cholesterol, insulin-resistant subjects display higher total cholesterol to HDL-cholesterol ratio, which suggests an increased risk of CVD. Total and LDL cholesterol concentrations were not significantly different between the 2 groups, but LDL particles were smaller. On the other hand, no significant differences were observed in plasma free cholesterol, cholesterol ester, and phospholipids between the 2 groups.

#### Intestinal Insulin Signaling

We first assessed phosphorylation of insulin signaling molecules in the intestine of obese subjects. Basal Ser473 phosphorylation of protein kinase B was reduced in the intestine of insulin-resistant compared with insulin-sensitive subjects (Figure 1A). This finding was accompanied with an increase in Thr180/Tyr182 phosphorylation of p38 mitogen-activated protein kinase involved in the signal transduction of stress and inflammatory cytokines in several metabolic pathways (Figure 1B). These observations suggest that systemic IR is associated with alterations in intracellular signaling of insulin and cytokines involved in key intestinal metabolic process.

#### Intestinal Oxidative Stress Markers

The presence of oxidative stress in the intestine of insulin-resistant subjects was assessed using biomarkers of lipid peroxidation. Using HPLC, we noted higher levels of

### Table: Nonstandard Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
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<tbody>
<tr>
<td>ABCA1</td>
<td>ATP-binding cassette A1 transporter</td>
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<tr>
<td>Apo B</td>
<td>apolipoprotein B</td>
</tr>
<tr>
<td>BMI</td>
<td>body mass index</td>
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<tr>
<td>CETP</td>
<td>cholesteryl ester transfer protein</td>
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<tr>
<td>CVD</td>
<td>cardiovascular disease</td>
</tr>
<tr>
<td>FABP</td>
<td>fatty acid-binding protein</td>
</tr>
<tr>
<td>HDL</td>
<td>high-density lipoprotein</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>homeostasis model assessment of insulin resistance (IR)</td>
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<tr>
<td>IR</td>
<td>insulin resistance</td>
</tr>
<tr>
<td>LDL</td>
<td>low-density lipoprotein</td>
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<tr>
<td>MTP</td>
<td>microsomal triglyceride transfer protein</td>
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<tr>
<td>NF-κB</td>
<td>nuclear factor-κB</td>
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<tr>
<td>PCSK9</td>
<td>proprotein convertase subtilisin/kexin type 9</td>
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<tr>
<td>T2D</td>
<td>type 2 diabetes mellitus</td>
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<tr>
<td>TRL</td>
<td>triglyceride-rich lipoprotein</td>
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</table>

**Nonstandard Abbreviations and Acronyms**

- PCSK9: proprotein convertase subtilisin/kexin type 9
- MTP: microsomal triglyceride transfer protein
- HDL: high-density lipoprotein
- BMI: body mass index
- CVD: cardiovascular disease
- IR: insulin resistance
- HOMA-IR: homeostasis model assessment of insulin resistance (IR)
- LDL: low-density lipoprotein
- NF-κB: nuclear factor-κB
- PCSK9: proprotein convertase subtilisin/kexin type 9
- T2D: type 2 diabetes mellitus
- TRL: triglyceride-rich lipoprotein
Table 1. Anthropometric and Glucose Homeostasis of Insulin-Sensitive and Insulin-Resistant Obese Subjects Matched for Age, Sex, and BMI

<table>
<thead>
<tr>
<th>Variables</th>
<th>Insulin-Sensitive Obese Subjects (n=10)</th>
<th>Insulin-Resistant Obese Subjects (n=10)</th>
<th>P Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anthropometrics</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sex</td>
<td>5 F/5 M</td>
<td>5 F/5 M</td>
<td>...</td>
</tr>
<tr>
<td>Age, y</td>
<td>45.0±10.0</td>
<td>40.3±10.1</td>
<td>NS</td>
</tr>
<tr>
<td>Weight, kg</td>
<td>138.3±21.4</td>
<td>150.0±31.3</td>
<td>NS</td>
</tr>
<tr>
<td>Height, cm</td>
<td>161.8±9.7</td>
<td>165.9±8.5</td>
<td>NS</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>54.0±7.3</td>
<td>53.7±7.6</td>
<td>NS</td>
</tr>
<tr>
<td>Glucose homeostasis</td>
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<td></td>
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<td>Glycemia, mmol/L</td>
<td>5.5±0.5</td>
<td>5.9±0.6</td>
<td>NS</td>
</tr>
<tr>
<td>Insulinemia, μU/mL</td>
<td>7.6±3.1</td>
<td>52.3±29.6</td>
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<td>HOMA-IR</td>
<td>1.9±0.8</td>
<td>13.9±8.7</td>
<td>0.002</td>
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<tr>
<td>Glycohemoglobin, %</td>
<td>5.7±0.2</td>
<td>6.0±0.3</td>
<td>0.02</td>
</tr>
</tbody>
</table>

Results are expressed as mean±SD, and paired t test P values are indicated. BMI indicates body mass index; F, female; HOMA-IR, homeostasis model assessment of insulin resistance; M, male; and NS, not significant.

Table 2. Lipid Profile of Insulin-Sensitive and Insulin-Resistant Obese Subjects Matched for Age, Sex, and BMI

<table>
<thead>
<tr>
<th>Variables</th>
<th>Insulin-Sensitive Obese Subjects (n=10)</th>
<th>Insulin-Resistant Obese Subjects (n=10)</th>
<th>P Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Triglycerides, mmol/L</td>
<td>1.14±0.48</td>
<td>1.90±0.48</td>
<td>0.0001</td>
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<tr>
<td>Phospholipids, mmol/L</td>
<td>0.94±0.17</td>
<td>1.00±0.18</td>
<td>NS</td>
</tr>
<tr>
<td>Total cholesterol, mmol/L</td>
<td>4.18±0.82</td>
<td>4.10±1.21</td>
<td>NS</td>
</tr>
<tr>
<td>Free total cholesterol, mmol/L</td>
<td>1.30±0.24</td>
<td>1.29±0.40</td>
<td>NS</td>
</tr>
<tr>
<td>Total cholesterol ester, mmol/L</td>
<td>2.88±0.71</td>
<td>2.81±0.90</td>
<td>NS</td>
</tr>
<tr>
<td>HDL-cholesterol, mmol/L</td>
<td>1.32±0.27</td>
<td>1.05±0.22</td>
<td>0.004</td>
</tr>
<tr>
<td>LDL-cholesterol, mmol/L</td>
<td>2.41±0.73</td>
<td>2.82±1.04</td>
<td>NS</td>
</tr>
<tr>
<td>Cholesterol/HDL-cholesterol</td>
<td>3.45±0.77</td>
<td>4.65±0.94</td>
<td>0.01</td>
</tr>
<tr>
<td>LDL particle size, Å</td>
<td>254.4±1.9</td>
<td>252.4±1.6</td>
<td>0.01</td>
</tr>
</tbody>
</table>

Results are expressed as mean±SD, and t test P values are indicated. BMI indicates body mass index; HDL, high-density lipoprotein; LDL, low-density lipoproteins; and NS, not significant.

observed in the activity of other antioxidant enzymes such as catalase (Figure 2D) and glutathione peroxidase (Figure 2E). A decreased superoxide dismutase activity may suggest a failure to properly protect the intestine against the negative impact of endogenous oxygen-free radicals and exogenous pro-oxidant conditions.

Intestinal Inflammatory Markers

Because low-grade inflammation is causative of IR, we determined the status of inflammatory markers in the intestine of insulin-resistant and insulin-sensitive subjects. Intercellular adhesion molecule-1 levels were higher in the intestinal samples of insulin-resistant subjects (Figure 3A). A rise in the concentrations of interleukin-6 was also found in the culture media of intestinal explants of insulin-resistant subjects (Figure 3B). Moreover, the addition of proinflammatory lipopolysaccharides to the organ culture further induced interleukin-6 secretion, and this phenomenon was significantly greater in the intestine of insulin-resistant compared with insulin-sensitive subjects (Figure 3B). Furthermore, the analysis of tumor necrosis factor-α revealed a rise in gene and protein expression although the latter reached a lesser statistical significance (P<0.06; Figure 2C and 2D). In addition, cyclooxygenase 2 protein content was significantly higher in the intestine of insulin-resistant compared with insulin-sensitive subjects (Figure 2E). Because most of the aforementioned inflammatory factors are activated by nuclear factor-κB (NF-κB), the transcription factor that initiates and amplifies inflammation, we finally assessed the activation of this pathway. We noticed a slight but non significant increase in NF-κB protein content, but the NF-κB to inhibitor of κB ratio, an indicator of NF-κB pathway activation, was significantly increased (Figure 2F). No significant change was observed in c-Jun N-terminal kinases pathway activation as well as in Toll-like receptor 4 and interleukin-1β gene expression between the intestinal samples of both groups of subjects (data not shown). These...
data suggest that the intestine of insulin-resistant subjects is more prone to develop a chronic low-grade inflammation state compared with the intestine of insulin-sensitive subjects.

**Intestinal De Novo Lipogenesis, Apo B-48 Biogenesis, and Lipoprotein Production**

To verify the impact of IR, oxidative stress, and inflammation on intestinal lipid metabolism, we measured de novo lipogenesis rate, Apo B-48 biogenesis, and TRL production. We found a significantly reduced intestinal Thr172 phosphorylation of 5′-adenosine monophosphate–activated protein kinase, which is indicative of its inactivation in insulin-resistant subjects (Figure 4A). In addition, Ser79 phosphorylation of acetyl-CoA carboxylase, a key downstream effector of 5′-adenosine monophosphate–activated protein kinase regulating lipid synthesis, was also reduced.

**Figure 2.** Evaluation of oxidative stress markers in the small intestine of insulin-sensitive (white bars) and insulin-resistant (black bars) obese subjects. Lipid peroxidation in tissue lysate was estimated using malondialdehyde (MDA; A) quantified as [MDA]-thiobarbituric acid complex by HPLC and conjugated dienes (B) measured by spectrophotometry. The amount of MDA and conjugated dienes in the tissue was normalized for total protein concentration. Fresh intestinal explants were incubated in the absence or presence of iron (0.2 mmol/L)-ascorbate (2 mmol/L; Fe2+/Asc; 1:10) system–mediated lipid peroxidation for 6 h before conjugated dienes quantification. The antioxidant activity of superoxide dismutase (SOD; C), catalase (D), and glutathione peroxidase (E) was measured in tissue lysate. The activity was normalized for total protein concentration. Data are mean±SEM for n=10 per group. *P<0.05 compared with insulin-sensitive subjects; #P<0.05 between control and Fe2+/Asc treatment of the same group.

**Figure 3.** Evaluation of inflammatory markers in the small intestine of insulin-sensitive (white bars) and insulin-resistant (black bars) obese subjects. Intercellular adhesion molecule-1 (ICAM-1; A) and interleukin-6 (IL-6; B) in tissue homogenates were quantified by ELISA. Values are expressed as picograms per milligram of protein. Fresh intestinal explants were incubated in the presence or absence of 100 μg/mL lipopolysaccharide (LPS) for 6 h. mRNA expression of tumor necrosis factor-α (TNF-α) gene was assessed by quantitative RT-PCR (C). The relative mRNA fold-changes between groups were calculated using the 2−ΔΔCt method. The results were normalized to ATP5O mRNA expression. Tissue homogenates were also analyzed by immunoblotting for the protein expression of TNF-α (D), cyclooxygenase-2 (Cox-2; E), and nuclear factor-kB (NF-kB) to inhibitor of NF-kB (IκB) ratio (F). Densitometric analyses of protein expression were normalized for protein expression levels of β-actin. Data are mean±SEM for n=10 per group. *P<0.05 compared with insulin-sensitive subjects; #P<0.05 between control and lipopolysaccharide treatment of the same group.
in the intestine of insulin-resistant compared with insulin-sensitive subjects (Figure 4C). The total expression levels of 5′-adenosine monophosphate–activated protein kinase α (AMPKα) at Thr172 (A), AMPKα (B), acetyl-CoA carboxylase (ACC) at Ser79 (C), and ACC (D). Densitometric analyses of protein expression were normalized for protein expression levels of β-actin. Intestinal de novo lipogenesis rates were measured by the incorporation of [1-14C]-acetic acid for 3 h (E). Data are expressed as nanomoles of acetic acid incorporated by a milligram of protein (insulin-sensitive, n=7; insulin-resistant, n=9). Apolipoprotein (Apo) B-48 synthesis by intestinal explants was evaluated by the incorporation of [35S]-methionine in immunopurified Apo B-48 resolved on SDS-PAGE acrylamide gel (F). Data are expressed as DPM of [35S]-methionine incorporated by a milligram of protein (insulin-sensitive, n=7; insulin-resistant, n=9). Triglyceride (TG)-rich lipoprotein (TRL) production by intestinal explants was evaluated by the incorporation of [1-14C]-oleic acid in TRL isolated by ultracentrifugation (G). Data are expressed as DPM of [1-14C]-oleic acid incorporated by a milligram of protein (insulin-sensitive, n=7; insulin-resistant, n=9). Data are mean±SEM (*P<0.05).

Figure 4. Intestinal lipid synthesis and lipoprotein biogenesis in the small intestine of insulin-sensitive and insulin-resistant obese subjects. Tissue homogenates were analyzed by immunoblotting for the phosphorylation of 5′-adenosine monophosphate–activated protein kinase α (AMPKα) at Thr172 (A), AMPKα (B), acetyl-CoA carboxylase (ACC) at Ser79 (C), and ACC (D). Densitometric analyses of protein expression were normalized for protein expression levels of β-actin. Intestinal de novo lipogenesis rates were measured by the incorporation of [1-14C]-acetic acid for 3 h (E). Data are expressed as nanomoles of acetic acid incorporated by a milligram of protein (insulin-sensitive, n=7; insulin-resistant, n=9). Apolipoprotein (Apo) B-48 synthesis by intestinal explants was evaluated by the incorporation of [35S]-methionine in immunopurified Apo B-48 resolved on SDS-PAGE acrylamide gel (F). Data are expressed as DPM of [35S]-methionine incorporated by a milligram of protein (insulin-sensitive, n=7; insulin-resistant, n=9). Triglyceride (TG)-rich lipoprotein (TRL) production by intestinal explants was evaluated by the incorporation of [1-14C]-oleic acid in TRL isolated by ultracentrifugation (G). Data are expressed as DPM of [1-14C]-oleic acid incorporated by a milligram of protein (insulin-sensitive, n=7; insulin-resistant, n=9). Data are mean±SEM (*P<0.05).
capacity to transport lipids and to produce TRL compared with the intestine of insulin-sensitive subjects. In addition, our data strengthen the hypothesis that fresh intestinal explants from insulin-resistant subjects have a higher capacity to produce TRL compared with explants from insulin-sensitive subjects.

**Intestinal Cholesterol Metabolism**

We examined the expression of genes involved in cholesterol metabolism to assess the intestinal involvement in the altered cholesterol phenotype observed in insulin-resistant subjects (Table 2). The protein amount of most cholesterol transporters (ie, NPC1L1, SR-B1, ABCG5, and ABCG8) seemed unchanged in the intestine of both groups of subjects. In contrast, we observed reduced ATP-binding cassette A1 transporter (ABCA1) mRNA expression in the intestine of insulin-resistant compared with insulin-sensitive subjects (Figure 6A). Importantly, this transporter mediates intestinal cholesterol efflux and is essential to the formation of HDL. Intestinal mRNA expression of cholesteryl ester transfer protein (CETP), a negative modulator of HDL-cholesterol, remained unchanged between insulin-sensitive and insulin-resistant subjects (Figure 6B). The reduced intestinal protein content of proprotein convertase subtilisin/kexin type 9 (PCSK9), involved in LDL receptor recycling, was also found in insulin-resistant compared with insulin-sensitive subjects (Figure 6C). Finally, we found lower levels of the activated form of sterol regulatory element–binding protein 2 in insulin-resistant subjects (Figure 6D). This latter factor is known to regulate the expression of genes maintaining cholesterol metabolism. Taken together, these results suggest that the intestine of insulin-resistant subjects seems to have a higher capacity to capture circulating LDL-cholesterol and a lower potential to released cholesterol into nascent HDL particles.

**Discussion**

If obesity and T2D are among the most important scourges of the 21st century, the common underlying driver seems to be IR, which is a prime etiologic factor for dyslipidemia and CVD. In fact, obesity has been proposed to predate and promote IR molecular defect(s), although this assumption has not been proven in humans and was not addressed particularly in the small intestine. Another important issue to clarify is whether a post-receptor defect, which reduces insulin sensing of the classical organs (including skeletal muscle, adipose tissue, and liver),
is related to oxidative stress and inflammation potentially residing in the gut of obese subjects. The final impetus consists in defining whether the combination of the aforementioned abnormal processes contributes to intestinal TRL overproduction and abnormal cholesterol metabolism. These puzzling and interesting hypotheses were tested by taking advantage of bariatric surgery, which offered us a unique opportunity to obtain fresh human intestinal specimens. The present experiments show that the duodenal tissue of patients with systemic IR was endowed with shabby insulin sensitivity as assessed by low protein phosphorylation of protein kinase B but high protein phosphorylation of p38 mitogen-activated protein kinase. The presence of oxidative stress was evidenced by increased malondialdehyde and conjugated diene levels, whereas the occurrence of inflammatory processes was highlighted by the sharp rise in intercellular adhesion molecule-1, cyclooxygenase-2, tumor necrosis factor-α, and interleukin-6 levels as well as in NF-κB activation. Impaired insulin sensitivity along with oxidative stress and inflammation likely impinge on lipid homeostasis as evidenced by the exaggerated lipogenesis and increased TRL assembly probably triggered by the stimulation of expression of I-FABP, L-FABP, Apo B-48, and MTP. The intestinal mucosa of insulin-resistant subjects also showed abnormalities in the expression of genes involved in cholesterol metabolism (ABCA1, PCSK9, sterol regulatory element–binding protein 2), suggesting that the gut may play a key role in the altered cholesterol phenotype observed in the presence of systemic IR. These results suggest that the small intestine could be classified as an insulin-sensitive tissue that may become resilient to insulin action in adverse metabolic conditions. Whereas, reduced insulin signaling, conceivably triggered by oxidative stress and inflammation, may prompt an exacerbated lipid and lipoprotein synthesis as well as an abnormal cholesterol profile and could, therefore, contribute to atherogenic dyslipidemia observed in subjects with metabolic syndrome and T2D.

Intriguingly, little attention has been paid to the role that human intestine could play in cardiometabolic disorders, though several observations allude to its active implication as a valuable actor equipped with insulin signaling/sensitivity and redox-inflammatory synergy. The intestine is the target of insulin-resistant subjects. These findings emphasize that metabolic conditions/stimuli induced the degradation of cytosolic inhibitor of NF-κB with the ensuing translocation of NF-κB to the nucleus, which transcriptionally modulates the cellular genes implicated in inflammatory responses in the gut of insulin-resistant subjects. Collectively, these data indicate that the oxidative stress machinery and inflammatory signaling are not only interrelated but their impairment can lead to an inhibition of insulin responses as well as a higher risk of CVD and associated factors in the small intestine of insulin-resistant obese patients. Currently, it is difficult to establish the temporal sequence of the complex and intimate relationship between increased oxidative stress and enhanced inflammation.

To our knowledge, we are the first group to report clear evidence of metabolic alterations in the small intestine of humans with systemic IR. Our results are generally in agreement with those obtained in previous studies performed in animal models with genetic and environment-induced obesity. Alongside the well-established role of the liver in the development of atherogenic dyslipidemia, our findings suggest that intestinal TRL overproduction play a key role in this pathology as shown in animal models. Indeed, the presence of an intestinal IR state has been previously suggested in fructose-fed hamster and in Psammomys obesus. The present study points out to the
local insulin signaling defects that may alter the physiological regulation of intestinal TRL production in obese insulin-resistant subjects. The overproduction of intestinal TRL may be a consequence of (1) higher intracellular fatty acid transport capacity by I-FABP and L-FABP, (2) increased de novo lipogenesis rates through higher acetyl-CoA carboxylase activity, (3) increased Apo B-48 biogenesis, and (4) enhanced Apo B-48 lipidation capacity by MTP. The present results and conclusions are strongly supported by the findings of increased de novo lipogenesis rate, increased expression of L-FABP, higher abundance of MTP protein expression, and reduced proteasomal degradation of Apo B-48 in the small intestine of insulin-resistant animals. Therefore, the present study presents possible mechanisms filling the gap between human in vivo observation and the detailed characterization of intestinal metabolism performed in animal models of IR.

In addition to these defects related to lipid metabolism and TRL production, we reported several molecular alterations indicating the presence of an aberrant intestinal cholesterol metabolism. We first analyzed the expression of apical cholesterol transporters (NPC1L1, ABCG5, and ABCG8) but could not detect any significant alterations, suggesting a similar capacity of the enterocyte to absorb luminal cholesterol in obese individuals with IR. In addition, the examination of ABCA1 located at the basolateral membrane showed a reduced expression, suggesting the low ability to efflux cholesterol to nascent HDL, which may explain the low levels of HDL-cholesterol in the plasma of insulin-resistant subjects.

Lower HDL-cholesterol has been described in several insulin-resistant or diabetic populations and has been recognized as an independent risk factor for CVD. Several studies have also reported the triglyceride enrichment of HDL, as a key mechanism leading to reduced HDL levels, in insulin-resistant and T2D patients. In fact, increased CETP in blood circulation enhanced the transfer of triglyceride from TRL to HDL and cholesterol esters from HDL particles to TRL. As a consequence, HDL triglyceride hydrolysis is accelerated and the resulting small and dense HDL particles are more rapidly cleared from blood circulation. Because the small intestine is known as an important contributor to circulating CETP, we tested the intestinal mRNA content of CETP in the 2 groups of obese subjects and did not observe differences. Therefore, the modulation of CETP in the gut is not implicated in the mechanisms that lower HDL-cholesterol levels in insulin-resistant obese individuals. It seems more plausible that the intestine is linked to this phenotype through the low intestinal ABCA1 expression.

In the present investigation, we also focused on PCSK9, a protease that posttranscriptionally enhances LDL receptor degradation, thereby regulating the level of LDL-cholesterol in blood circulation. Conversely, the inactivation of PCSK9 enhances the number of LDL receptor molecules on the cell surface and accelerates the clearance of circulating LDL-cholesterol. Although most of the studies have emphasized the central role of PCSK9 in liver cholesterol metabolism, growing evidence documents similar PCSK9 actions in the small intestine. Although PCSK9 protein expression is strongly downregulated by high cholesterol concentrations and diabetes mellitus induction in the 2 organs, the circulating levels of PCSK9 were not altered in the presence of systemic IR and T2D. In the present investigation, PCSK9 expression was reduced in the intestine of insulin-resistant subjects, which is in line with diabetic data previously reported. However, additional investigation is absolutely required given the growing evidence related to the activation of the assembly of TRL synthesis in the liver and intestine.

Using the present methodological approach, we can only associate the overall capacity of the intestine to transport lipids and to produce TRL with the presence of IR independent of the influence of luminal lipid composition. This represents a strength of our study because we directly assessed the impact of local IR on intestinal metabolism without the interference of lipid composition. However, the experimental approach constitutes a limitation because the impact of lipid composition may represent a major determinant of lipid metabolism in insulin-resistant subjects. A detailed food assessment was not available for these subjects, but we observed some differences in plasma fatty acid composition measured by gas chromatography. The results showed increased total fatty acid concentrations as well as elevated saturated fatty acid proportion with reduced percentage of polyunsaturated fatty acids, which may expose the intestinal tissue to inadequate fatty acid pattern and may thus exacerbate inflammation, oxidative stress, and lipid metabolism in the gut. More studies are required to analyze the composition of fatty acids in the intestinal tissues of insulin-resistant obese subjects.

The insulin-sensitive versus insulin-resistant subjects who participated in the present investigation were paired for age, sex, and BMI. However, we acknowledge some limitations of the study, including its cross-sectional design that prevented inference about a cause-and-effect relationship between the presence of intestinal IR, oxidative stress, chronic low-grade inflammation, and development of metabolic alterations leading to intestinal overproduction of TRL in insulin-resistant subjects. The difficulty in obtaining intestinal tissue from these subjects limited our capacity to perform a longitudinal study and to definitively conclude on the chronology of intestinal versus systemic metabolic dysregulation. Future studies are needed to deeply investigate the impact of intestinal IR on cholesterol and lipid metabolism in humans. A direct induction of IR in fresh biopsies from insulin-sensitive subjects could be a possible approach to test this issue.

In conclusion, we demonstrated that the human small intestine should be considered an insulin-sensitive tissue that can be deregulated by the occurrence of local oxidative stress and inflammation. These alterations are likely associated with a pronounced increase in lipid synthesis and Apo B-48 biogenesis as well as with an increased TRL assembly and secretion. These observations point out to the potential mechanisms that link small intestine metabolism to the development of atherogenic dyslipidemia commonly observed in subjects with metabolic syndrome and T2D. Further studies are needed to better define the role of small intestine in atherogenic dyslipidemia because it may help in identifying new therapeutic strategies to reduce CVD risk in insulin-resistant and diabetic patients.

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Disclosures

None.

References

Intestinal Lipid Metabolism in Insulin Resistance

This study demonstrates that the small intestine develops an insulin resistance state in obese subjects with systemic insulin resistance (IR). The local insulin resistance seems to be engendered by a marked pro-oxidative and proinflammatory intestinal environment. These alterations support a novel mechanism for diabetic dyslipidemia evidenced by a pronounced increase in lipid synthesis and TRL secretion by the small intestine of obese subjects with systemic IR. To our knowledge, this study is the first to directly assess small intestine metabolic states in humans with systemic IR. Few studies have evaluated the role of intestine in diabetic dyslipidemia, and most of them were performed in animal models. The results of the present study in humans are, thus, a significant contribution to fill the gap between human in vivo observations and detailed mechanistic studies of intestinal lipid metabolism performed in animal models of IR.

Significance
Intestinal Lipid Handling: Evidence and Implication of Insulin Signaling Abnormalities in Human Obese Subjects
Alain Veilleux, Émilie Grenier, Picard Marceau, André C. Carpentier, Denis Richard and Emile Levy

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SUPPLEMENTAL MATERIALS AND METHODS

Subjects and tissue sampling
The main study sample included severely obese subjects (BMI ≥ 40 kg/m²), without T2D, undergoing biliopancreatic diversion at the Quebec Heart and Lung Institute, Laval University (Quebec City, Canada). Insulin and glucose levels were assessed in the plasma of each subject to calculate the homeostatic model assessment of insulin resistance (HOMA-IR). Twenty of these subjects (10 women and 10 men) were assigned to two groups matched for age (±10 years), gender and body mass index (BMI) (±5 units), but with either relatively low (<3) or high (>7) IR according to their HOMA-IR. None of these subjects received a therapy with estrogen, hypoglycaemic agents and cholesterol- or TG-lowering agents. Duodenum specimens were obtained during the surgery. The intestinal samples were immediately transferred to the laboratory for functional studies (e.g. de novo lipogenesis, apo B-48 biogenesis and chylomicrons assembly and secretion). The remaining tissue was immediately frozen in liquid nitrogen and stored at –80 °C for subsequent analyses. Written informed consent was obtained from all subjects. The project was approved by the ethics committees of Quebec Heart and Lung Institute, Laval University (Quebec City, Canada) and Sainte-Justine Research Center (Montreal, Canada).

Anthropometric, lipid profile and glucose homeostasis
BMI was measured on the morning of the surgery. Similarly, overnight fasting blood samples were drawn on the morning of the surgery. Total cholesterol, free cholesterol and triglyceride levels were estimated in the plasma by colorimetric enzymatic kits (Roche Diagnostic, Indianapolis, USA). Glucose was assessed using the glucose oxidase method and insulin was quantified with the ultrasensitive insulin assay on the Access® immunoassay system (Beckman Coulter, Brea, USA). The HOMA-IR index was calculated using the following formula: fasting insulin (µU/mL) x fasting glucose (mmol/L) ÷ 22.5.¹

Protein expression analysis
Intestinal samples were homogenized with a polytron in the lysis buffer (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄, 1.47 mM KH₂PO₄ (pH 7.4), 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 5 µg/ml leupeptin, 2 mg/ml pepstatine). Homogenates were agitated at 4°C for 1h in presence of 1% Triton and then centrifuged at 13,000 rpm for 10 min. The Bradford assay (Bio-Rad Laboratories, Mississauga, Canada) was used to determine protein concentration. Homogenates were prepared for Western blotting as described previously.² Same protein amounts (30 ug) were loaded on the SDS-PAGE and the β-actin protein was used as an internal control. The following antibodies (directed against human) and dilutions (1:1000 unless otherwise specified) were employed: mouse anti-β-actin (1:40000, Sigma Aldrich, St. Louis, USA); anti-Akt
(#ab32902) and anti-TNF-α (#ab66579) from Abcam (Cambridge, USA); anti-intestinal-fatty acid binding protein (I-FABP) and anti-liver-fatty acid binding protein (L-FABP) antibodies were raised in rabbits after injection of recombinant proteins;\(^3\) anti-microsomal transfer protein (MTP) was kindly provided by John Wetterau and Harris Jamil (Bristol-Myers Squibb Research Institute, USA);\(^4\) anti-SAR-1B (1:2000) was kindly provided by Randy Schekman (University of California, USA);\(^4\) anti-PCSK9 was kindly provided by Geneviève Dubuc and Jean Davignon (Clinical Research Institute of Montreal, Canada);\(^5\) anti-nuclear factor kappa B (NF-kB) p65 subunit (sc-372G) and anti-I-KappaB-alpha (IKB-α) (sc-1643) were obtained from Santa-Cruz Biotechnology (Santa-Cruz, USA); anti-phospho-Akt Ser473 (#9271), anti-phospho-AMPKα Thr172 (#40H9), anti-AMPKα (#2532), anti-phospho-p38 MAPK Thr180/Tyr182 (#4631), anti-p38 MAPK (#9212), anti-phospho-JNK Thr183/Tyr185 (#9251), anti-JNK (#9252), anti-phospho-Acetyl-CoA Carboxylase (ACC) Ser79 (#3661) and anti-ACC (#3662) were obtained from Cell Signaling Technology (Boston, USA).

**Messenger RNA expression**

Total RNA was isolated from intestinal tissue using the RNeasy lipid tissue extraction kit and on-column DNase treatment following the manufacturer’s recommendations (Qiagen, Valencia, USA). RNA quality was assessed using the Agilent Technologies 2100 bioanalyzer (Agilent, Santa Clara, USA). Complementary DNA was generated using the Superscript first strand synthesis system (Invitrogen, Carlsbad, USA). Real-time cDNA amplification was performed in duplicate using SYBR Green with the 7500 Real-Time PCR System (Applied Biosystems, Foster City, USA) and was carried out for 40 cycles. Target genes amplifications were normalized using expression levels of ATP synthase 5 subunit O (ATP5O). The relative mRNA fold changes between groups were calculated using the $2^{-\Delta\Delta Ct}$ method. Primer sequences used for each gene are shown in the following Table. Validation was performed to ensure optimal amplification efficiency of the target and reference gene cDNA.

**Table 1: Primer sequences used for quantitative RT-PCR**

<table>
<thead>
<tr>
<th>Target genes</th>
<th>Sense primers</th>
<th>Anti-sense primers</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABCA1</td>
<td>TGCTGTGGAAGAACCTCACCTT</td>
<td>CGAACAGAGATCAGGATCAGG</td>
</tr>
<tr>
<td>CETP</td>
<td>CTCTGCCATTGACCTCCAGA</td>
<td>AGGTAGCAGTCAGGGGCATC</td>
</tr>
<tr>
<td>TNFA</td>
<td>CCCCCAGGGACCTCTCTCTAA</td>
<td>TGGGCTACAGGGTTGTCCTA</td>
</tr>
<tr>
<td>IL1B</td>
<td>AGCCATGGCAGAAGTACCTTG</td>
<td>GGCCATCAGGTTCAAAGAAC</td>
</tr>
<tr>
<td>TLR4</td>
<td>AGAACTGCAGTGCTTGATT</td>
<td>ATGCCCCATCTTTCAATGTC</td>
</tr>
<tr>
<td>ATP5O</td>
<td>GCGATGCTTACGTACCTCTG</td>
<td>TGGCATAGGCACCTTCAATA</td>
</tr>
</tbody>
</table>
Lipid peroxidation appraisal

The amount of malondialdehyde (MDA) in tissue homogenates was determined by HPLC as described previously. Briefly, proteins were precipitated with sodium tungstate (Na2WO4) and the supernatant was incubated with thiobarbituric acid (TBA) at 90 °C for 60 min. The TBA2-MDA chromogen was extracted with 1-butanol, dried under nitrogen, resuspended in KH2PO4/methanol (70:30, pH 7.0) mobile phase before detection by HPLC coupled to a fluorescent detector. Conjugated dienes were extracted from tissue homogenate using heptane/isopropyl alcohol (2:1) and measured spectrophotometrically in heptane layer. Results were normalized for total protein concentration.

Antioxidant enzymes

Activities of the antioxidant enzymes were measured in tissue homogenates using adapted protocols from Pippenger et al. For catalase activity, tissue homogenates (10 µg) were mixed with phosphate-buffered saline (PBS) solution, pH 7.0, 10 mM hydrogen peroxide (H2O2) and absorbance was monitored by spectrophotometry at 240 nm for 3 min. For glutathione peroxidase (GPx) activity, tissue homogenates (150 µg) were incubated with PBS solution, pH 7.0, containing 1 mM glutathione (Sigma Aldrich, St. Louis, USA), 0.6 units of glutathione reductase (Sigma Aldrich, St. Louis, USA), and 100 mM β-nicotinamide adenine dinucleotide 2′-phosphate-reduced (NADPH) tetrasodium salt (Sigma Aldrich, St. Louis, USA) and 1.5% H2O2. Absorbance was monitored at 340 nm for 5 min. To assess superoxide dismutase (SOD) activity, tissue homogenate (130 µg) were incubated in presence of superoxide radicals (O2·−), which were generated by the addition of xanthine and xanthine oxidase (Sigma Aldrich, St. Louis, USA), and oxidation was followed by spectrophotometry at 550 nm for 5 min. All experiments were normalized for total protein concentration.

Inflammation markers

The levels of intercellular adhesion molecule-1 (ICAM-1) and Interleukin-6 (IL-6) were determined using ELISA kits from Abcam (Cambridge, USA) and from RayBiotech (Norcross, USA), respectively. Assays were performed according to the manufacturer’s protocol. Results were normalized for total protein concentration.

Intestinal organ culture

Fresh intestinal specimens were cleared of mesentery, split longitudinally, washed in culture medium, and cut into explants (3 × 7 mm). Explants were randomly transferred onto lens paper, with the mucosal side facing up, in organ culture dish (Falcon Plastics, Los Angeles, USA). Explants were cultured in serum-free Leibovitz L-15 medium with anti-proteases and antibiotics according to the technique described previously. To determine the responsiveness of fresh intestinal explants to oxidative stress, they were exposed to iron (0.2 mM)-
ascorbate (2 mM) (1:10) system-mediated lipid peroxidation. At the end of a 6 h culture period, the degree of lipid peroxidation was evaluated by measuring conjugated dienes in tissue homogenates. Similarly, fresh intestinal explants were incubated with lipopolysaccharides (LPS, 100 μg/ml, Sigma Aldrich, St. Louis, USA) for 6 h to examine the inflammatory response by assessing IL-6 secretion. All experiments were normalized for total protein concentration. At the end of explant incubation, integrity of the intestinal mucosa was examined (n=10/group) by assessing various variables related to cell proliferation ([³H]-thymidine incorporation), differentiation (sucrase), function (lactase, maltase), glucose metabolism (uptake and oxidation of glucose), cell viability and membrane integrity (leakage of lactate dehydrogenase and alkaline phosphatase to the medium) as assessed previously.

Table 2: Integrity assessment of intestinal explants in culture.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Insulin-sensitive obeses</th>
<th>Insulin-resistant obeses</th>
<th>P values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactase (μmol/min/g protein)</td>
<td>2.4 ± 0.4</td>
<td>2.6 ± 0.7</td>
<td>NS</td>
</tr>
<tr>
<td>Sucrase (μmol/min/g protein)</td>
<td>41.2 ± 5.5</td>
<td>39.1 ± 6.2</td>
<td>NS</td>
</tr>
<tr>
<td>Maltase (μmol/min/g protein)</td>
<td>99.0 ± 11.6</td>
<td>113.4 ± 14.8</td>
<td>NS</td>
</tr>
<tr>
<td>Lactate dehydrogenase (U/L)</td>
<td>213.9 ± 17.3</td>
<td>241.4 ± 19.8</td>
<td>NS</td>
</tr>
<tr>
<td>Alkaline phosphatase (μmol/min/g protein)</td>
<td>39.9 ± 4.1</td>
<td>36.2 ± 5.6</td>
<td>NS</td>
</tr>
<tr>
<td>Glucose uptake (% of total radioactivity)</td>
<td>27.8 ± 5.4</td>
<td>25.7 ± 6.3</td>
<td>NS</td>
</tr>
<tr>
<td>CO₂ production (% of total radioactivity)</td>
<td>3.8 ± 0.6</td>
<td>3.4 ± 0.5</td>
<td>NS</td>
</tr>
<tr>
<td>[³H]-thymidine incorporation (dpm/µg DNA)</td>
<td>295 ± 18</td>
<td>286 ± 22</td>
<td>NS</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± SD (n=10) and t-test p values are indicated. NS: Not significant.

De novo lipogenesis assay
Intestinal explants were incubated in the presence of 1 μCi of [1-14C]-acetic acid (Perkin Elmer, Waltham, USA) for 3 h. After incubation, explants were homogenized in PBS containing 1% (vol/vol) Triton X-100. Total lipids were extracted with 2:1 (vol/vol) chloroform-methanol. Solvents were dried under nitrogen gas, and resuspended in chloroform to assess incorporation of [1-14C]-acetic acid into lipid by liquid scintillation counting.

Apo B-48 and triglyceride-rich lipoproteins synthesis
After a 30-min stabilization period, intestinal explants were incubated in presence of a micellar mixture (6.6 mmol/l sodium taurocholate, 1 mmol/l oleic acid, 0.5 mmol/l monoolein, 0.1 mmol/l cholesterol, and 0.6 mmol/l phosphatidylcholine) containing 0.5 μCi of [1-14C]-oleic acid (specific activity: 53.9 mCi/mmol) or 300...
μCi $[^{35}S]$methionine (specific activity: >1000Ci/mmol) purchased from Perkin Elmer (Waltham, USA). Intestinal explants were cultured for 3 h at 37°C in an atmosphere of 5% CO₂. Determination of secreted triglyceride-rich lipoproteins (TRL) was performed by spinning the medium at 100,000g for 2.26 h at a density of 1.006 g/ml as described previously. Radioactivity in each fraction was measured by liquid scintillation counting. For determination of apo B-48 synthesis and secretion, the medium was incubated with excess anti-apo B polyclonal antibodies and protein A/G for 18 h at 4°C. The immunoprecipitates were separated using a linear 4–20% acrylamide gradient gel and counted by liquid scintillation as described previously.

**Statistical analyses**

Differences in parameters between groups were tested using paired t-test and repeated measures analysis of variances. Relative mRNA expression values ($\Delta\Delta Ct$) between groups were compared using the Wilcoxon signed-rank test. Differences were considered to be statistically significant at $p<0.05$. Statistical analyses were performed with JMP software (SAS Institute, Cary, USA)
REFERENCES


