Both Insulin Resistance and Diabetes in Psammomys obesus
Upregulate the Hepatic Machinery Involved in Intracellular VLDL Assembly

M. Zoltowska, E. Ziv, E. Delvin, M. Lambert, E. Seidman, E. Levy

Objective—In the current study, we examined the mechanisms that regulate hepatic apolipoprotein B (apoB)–containing lipoprotein secretion in Psammomys obesus, a good animal model for the investigation of insulin resistance and diabetes.

Methods and Results—When fed chow ad libitum, 22% maintained normoglycemia and normoinsulinemia (group A), 33% exhibited normoglycemia and appreciable hyperinsulinemia (group B), and 45% developed overt diabetes (group C). Body weight gain, plasma free fatty acid elevation, hypertriglyceridemia, and hypercholesterolemia characterized groups B and C. Triton WR-1339 injection, at fasting, resulted in higher plasma VLDL-triglyceride and VLDL-apoB accumulation in groups B and C, suggesting increased VLDL production by the liver. Pulse-chase labeling experiments in cultured hepatocytes with [35S]methionine revealed reduced intracellular degradation and enhanced secretion of newly synthesized apoB in groups B and C. Concomitant with the raised triglyceride and cholesterol contents in the livers of groups B and C, there was an increase in lipogenesis and in the activity of microsomal triglyceride transfer protein, monoacylglycerol acyltransferase, and diacylglycerol transferase. Pretreatment of hepatocytes with proteasomal inhibitors eliminated the differences in apoB secretion among groups A, B, and C.

Conclusions—Our data indicate that both insulin resistance and diabetes triggered the intracellular machinery involved in VLDL assembly and secretion. (Arterioscler Thromb Vasc Biol. 2004;24:118-123.)

Key Words: insulin resistance • diabetes • VLDL production • apo B degradation • microsomal triglyceride transfer protein

Non–insulin-dependent diabetes mellitus is a major cause of morbidity and mortality in industrialized nations, where it has reached epidemic levels.1 It is characterized by peripheral insulin resistance, increased hepatic glucose production, and defects in insulin secretion from pancreatic β-cells.2 The most commonly recognized lipid abnormality in this disorder is hypertriglyceridemia, which is known to be an independent risk factor for coronary heart disease.3 Though still a matter of debate, a hypertriglycerideremic state can be produced through 2 mechanisms: increased synthesis of VLDL or decreased removal of triglyceride (TG)-rich lipoproteins. The overproduction of VLDL is probably caused by an enhanced hepatic influx of free fatty acids (FFAs) or by the inability of insulin to inhibit the release of VLDL from the liver.4,5 However, additional studies are necessary to unveil the mechanisms underlying increased VLDL production in insulin resistance and type 2 diabetes.

Animal models are required for the study of the mechanisms behind insulin resistance and type 2 diabetes and for a better understanding of diabetes complications in human populations. Prominent among these animal species is the Israeli desert gerbil, Psammomys obesus (sand rat), which appears to be an ideal natural model of the disease in humans because it shows an increased tendency to develop diet-induced diabetes, which is associated with moderate obesity.6–8 The results indicate that the metabolism of Psammomys is well adapted toward life in a low-energy environment, where Psammomys takes advantage of its capacity for constant accumulation of adipose tissue for maintenance and breeding in periods of scarcity.6–8 This metabolism, known as “thrifty metabolism,” is activated when nutrient intake is high.8 Examples can be found among the Pima Indians, Australian aborigines, and many other Third World populations.9,10 Therefore, we consider that Psammomys with insulin resistance and hyperglycemia might be very useful for the study of hypertriglycerideremia mechanisms.

Apolipoprotein B (apoB) is an essential structural protein for the assembly of lipoproteins by the liver and intestine.11 ApoB-48, designated as the percentage of the N-terminus of the larger secretory product apoB-100, is produced from a single-gene transcript by mRNA editing and proteolytic cleavage.12–14 The regulation of apoB hepatic secretion rate is
posttranscriptional. Only a portion of de novo synthesized apoB is secreted, whereas the remaining portion is degraded intracellularly. Insulin-stimulated apoB degradation might involve phosphatidylinositol 3-kinase, translocation to the endoplasmic reticulum membrane, and the sorting of apoB to a post–endoplasmic reticulum degradative pathway without changing total apoB mRNA levels. We have used the Israeli desert gerbil Psammomys obesus in the present study in our search for the molecular background of the mechanisms that mediate the overproduction of VLDL in the insulin-resistant state resulting from overnutrition. Utilization of this animal model will help document the relevance of the nutritionally induced insulin resistance and diabetes in the intracellular events leading to TG-rich lipoprotein overproduction.

Methods

Please see the online data supplement, available at http://atvb.ahajournals.org.

Results

Animal Model Characteristics

When the animals were fed chow ad libitum, 22% maintained normoglycemia and normoinsulinemia (group A), 33% exhibited normoglycemia and appreciable hyperinsulinemia (group B), and 45% developed overt diabetes (group C) (Figure I, available online at http://atvb.ahajournals.org). Body weight gain and elevated FFA values characterized the animals of groups B and C (Figure II, available online at http://atvb.ahajournals.org). An increase in body weight is noteworthy despite the absence of hyperphagia documented by similar food consumption expressed either as grams per day (group A, 15.0±0.5; group B, 14.7±0.8; and group C, 14.6±0.7) or as grams per 100 g of animal weight (group A, 7.0±0.6; group B, 6.7±0.8; and group C, 6.6±0.9).

Plasma Lipids and In Vivo Hepatic VLDL Secretion

The spectrum of lipid profiles ranged from normal lipemia in group A to hypertriglycerideremia and hypercholesterolemia in groups B and C (Figure I). Furthermore, lipogenesis (micrograms of 1H incorporated per hour per gram) in groups B (31.5±4.9) and C (38.6±6.1) was significantly higher (P<0.05) than in that of group A (20.1±3.4). To determine whether enhanced VLDL secretion by the liver contributed to the high levels of plasma TG, Triton WR-1339 was injected to block the peripheral removal of newly secreted VLDL while simultaneously monitoring the rate of TG accumulation in vivo. The administration of Triton WR-1339 resulted in the accumulation of VLDL-TG in all animal groups (Figure 1A). However, the VLDL-TG secretion rate in groups B (38.4%, P<0.01) and C (47.5%, P<0.005) was significantly higher than in group A. Similarly, the rate of total VLDL-apoB production was significantly increased by 99.9% (P<0.001) in group B and by 168% (P<0.001) in group C compared with controls (Figure 1B).

In Vitro ApoB Synthesis

To define the mechanisms of insulin resistance, and diabetes-stimulated secretion of apoB in Psammomys obesus, we used intact hepatocytes. First, hepatocytes were pulsed with [35S]methionine for 10 minutes and chased for 90 minutes. As shown in Figure 2, there was an apparent degradation of apoB in all animal groups before any significant secretion of apoB occurred. Indeed, when the values from the 10-minute-chase time point were taken as 100% and the cellular content of radiolabeled apoB was monitored for an additional 90 minutes of chase with excess cold methionine, they indicated a continuous fall in the relative amount of immunoreactive apoB-48 and apoB-100 in the hepatocytes of all animal groups. However, there was much less degradation of newly synthesized apoB in groups B and C than in group A. Radiolabeled apoB was detected in the medium after 30 minutes of chase. Nevertheless, the level of [35S]-labeled apoB recovered from group B and C media was always higher than that from group A media. Besides, no appreciable effects were observed in de novo albumin synthesis (results not shown). The amount of radiolabeled albumin immunoprecipitated from the cells (quantified by cutting and scintillation counting of the bands) was similar among hepatocytes derived from groups A, B, and C. This again confirmed the notion that these conditions do not alter the specific synthesis of individual proteins such as albumin. Overall, our pulse-chase experiments have documented that insulin resistance and diabetes increased the secretion of newly synthesized apoB by inhibiting its degradation in the hepatocytes of Psammomys obesus.

MTP Activity

To further explore the putative effects of insulin resistance and diabetes on microsomal triglyceride transfer protein (MTP) activity, the livers of Psammomys obesus were subjected to subcellular fractionation and microsome isolation. Analysis of hepatic TG transfer activity revealed an increase in group B (46% P<0.006) and group C (56% P<0.0003) compared with control animals. (Figure III, available online
Pulse-chase analysis of intracellular and secreted apoB. Hepatocytes were pulse-labeled with [35S]methionine for 1 hour and chased for periods of 1, 2, 4, and 16 hours in the presence of 10 mM excess cold methionine. 35S-labeled apoB was immunoprecipitated from cell homogenates and from the chase medium and analyzed by SDS-PAGE. Bands corresponding to apoB-48 and apoB-100 were counted after digestion. Values are mean±SE for n=5 per group. *P<0.05 vs group A.

Hepatic Lipid Synthesis and Liver Enzyme Activities

In the next step, we quantified the lipid content of the Psammomys obesus livers. The concentrations of TG, cholesterol, and phospholipids were higher in groups B and C than in the control group A (Table 1, available online at http://atvb.ahajournals.org). Thus, the provision of lipids might direct apoB into the secretion pathway. Because both GPAT and DGAT are considered regulatory enzymes in the liver, we determined their activity in isolated microsomes. The specific activity of GPAT remained relatively unchanged among groups A, B, and C. However, DGAT activity significantly increased in group B and displayed an elevated trend in group C compared with group A. Noteworthy is the increase in DGAT, which is consistent with TG biosynthesis and accumulation, as well as VLDL production by the liver in groups B and C (Figure IV, available online at http://atvb.ahajournals.org). Similarly, the specific activity of MGAT was increased in groups B and C (Figure IV), suggesting that the monoaocylglycerol pathway of glycerolipid synthesis could also be induced in the insulin-resistant and diabetic liver of Psammomys obesus.

Effects of Proteasome Inhibitors on VLDL-ApoB Secretion

<table>
<thead>
<tr>
<th>Animal Group</th>
<th>A</th>
<th>B</th>
<th>C</th>
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</thead>
<tbody>
<tr>
<td>Control</td>
<td>1505±293</td>
<td>3629±433</td>
<td>3221±536*</td>
</tr>
<tr>
<td>ALLN</td>
<td>4471±382*</td>
<td>4834±197*</td>
<td>5036±371*</td>
</tr>
<tr>
<td>Lactacystin</td>
<td>4380±410*</td>
<td>4902±582</td>
<td>4957±280*</td>
</tr>
</tbody>
</table>

Primary hepatocytes isolated from Psammomys obesus liver were preincubated with N-acetyl-leucyl-norleucin (ALLN, 40 μg/mL) and lactacystin (1 μmol/L). They were then pulsed with 300 μCi/mL [35S]methionine for 2 hours in the presence of the proteasome inhibitors and 0.8 mM oleic acid complexed to albumin. The media samples were subjected to ultracentrifugation. The VLDL samples were reacted with a specific anti-Psammomys obesus apoB antibody for 18 hours at 4°C. At the end of this reaction, Pansorbin was added, and the mixture was reincubated at 20°C for 60 minutes. The immunoprecipitates were washed and analyzed by SDS-PAGE. Bands corresponding to apoB-48 and apoB-100 were sectioned, pooled, and counted after an overnight incubation at 20°C with BTS-450 and 10 mL liquid scintillation fluid.

Values are mean±SE, expressed as dpm/mg protein for 3 experiments. *P<0.05 vs corresponding control in each group.

VLDL-ApoB Secretion and Proteasome Inhibition

To determine whether proteasome inhibition affected the secretion of VLDL-apoB, primary hepatocytes isolated from Psammomys obesus were treated with powerful proteasome inhibitors. Without proteasome inhibitor supplementation, there was a significant elevation in the amount of VLDL-apoB secreted into the media by hepatocytes from group B and C animals (Table I), which thus confirmed the in vivo Triton WR-1339 studies (Figure I). These observations suggested that the radiolabeled apoB content of hepatocytes from group A disappeared more rapidly, and they conversely supported the enhanced efficiency of VLDL assembly and secretion in hepatocytes from groups B and C. The addition of N-acetyl-leucyl-norleucin (40 μg/mL) and lactacystin (1 μmol/L) led to the stimulated incorporation of [35S]methionine into VLDL-apoB released from hepatocytes of all animal groups compared with untreated cells (Table 1). Nevertheless, the percentage increase in group A allowed the hepatocytes to catch up to the values observed in groups B and C. Thus, it is reasonable to conclude that the hepatic overproduction of apoB noted in insulin resistance and diabetes might be caused by the reduced degradation of apoB.

Insulin Resistance Parameters

Our final experiments were aimed at determining whether the differences observed between groups B and C were due to the degree of insulin resistance or to the development of hyperglycemia per se. The measurement of de novo glucose synthesis in hepatocytes incubated with [14C]alanine revealed higher gluconeogenesis in group C than in group B (Figure 3). Similarly, the uptake of [3H]-2-deoxyglucose by the soleus was increased in group C over that in group A (Figure 3). Furthermore, protein kinase Cε was found to be more overexpressed in the soleus of group C, whereas Akt and GLUT4 proteins were decreased (Figure 4). Finally, to define better the insulin resistance status, a euglycemic hyperinsulinemic clamp was carried out in the 3 groups of Psammomys obesus.
During insulin infusion, there were no significant differences in plasma insulin and glucose concentrations among groups at each step of the clamp (results not shown). Plasma glucose levels were clamped at baseline levels (9.6 mmol/L). The rate of glucose infusion necessary to maintain euglycemia was significantly lower in groups B (36.7%) and C (80.6%) versus group A (Figure 5). A similar pattern was observed in the glucose disappearance rate. Additionally, compared with the control group A, groups B (33.3%) and C (66.7%) also displayed higher glucose production rate (EGP). As noted from these euglycemic hyperinsulinemic clamp studies, group B *Psammomys obesus* had higher values of glucose infusion rate (GIR) and glucose disappearance rate (Rd) as well as lower values of EGP than did group C animals, suggesting greater insulin resistance (Figure 5).

**Discussion**

The gerbil *Psammomys obesus* offers an attractive model for investigating the interaction between both insulin resistance and diabetes with the mechanisms of hepatic TG-rich lipoprotein overproduction. Interestingly, its diabetic phenotype revealed itself during transfer from its native salt bush (*Atriplex halimas*) diet to a laboratory rodent diet, which is higher in energy (3.1 kcal/g) than is its native desert food (1.9 kcal/g).19,20 Our studies were able to demonstrate that hepatic VLDL-TG and VLDL-apoB secretion contribute to characteristic hypertriglyceridemia in this interesting animal model. First, we used Triton WR-1339, a nonionic detergent that coats VLDL particles, thus blocking the action of lipoprotein lipase in vivo and preventing VLDL catabolism.21 The accumulation of plasma TG-rich lipoproteins after Triton treatment reflects the TG secretion rate from the liver and intestine. Because the animals were fasted during this experiment, one could assume intestinal contribution to be minimal with TG output mainly from the liver. However, recent data have shown a production of intestinally derived lipoprotein particles even in the fasting state.22

Subsequently, pulse-chase experiments with cultured hepatocytes confirmed that insulin resistance and diabetes led to a significant increase in apoB secretion in *Psammomys obesus*. Raised availability of neutral lipids as well as augmented MTP activity resulted in apoB degradation and secretion. The enhanced apoB output in *Psammomys obesus* likely reflects adequate lipidation of apoB by MTP, a critical player in the early posttranslational regulation of apoB.23 Accordingly, genetic MTP deficiency has been shown to be the basis for the lack of plasma apoB in patients with abetalipoproteinemia.24 Similarly, knockout mice exhibited a marked reduction in plasma apoB concentrations because of the severely impaired hepatic secretion of apoB-containing lipoproteins.23 MTP inhibitors also led to a substantial reduction in MTP activity, with a lowering of plasma lipoprotein levels.26 Conversely, increased secretion of VLDL-TG and VLDL-apoB was noted in mice with adenovirus-induced hepatic overexpression of MTP.27 Likewise, MTP activity and in vivo TG secretion were augmented in the hamster.

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**Figure 3.** Liver gluconeogenesis and soleus glucose uptake. Gluconeogenesis in hepatocytes from 12-hour-fasted animals was measured by the determination of glucose production from [14C]alanine after a 20-minute incubation (A). Glucose uptake by the soleus muscle was evaluated by adding [3H]-2-deoxyglucose to the medium (B). Data represent mean±SE for n=5 per group. *P<0.05 vs group A. **P<0.01 vs group A.

**Figure 4.** Expression of protein kinase (PK) Cε, Akt, and GLUT4 in the soleus. To determine PKCε and Akt, muscle lysates were subjected to SDS-PAGE followed by Western blotting and then probed with antibodies. Immunoreactive proteins were made visible with horseradish peroxidase–coupled secondary antibodies and enhanced chemiluminescence. For expression of GLUT4, soleus membranes were isolated, homogenized, loaded on SDS-PAGE gels, and immunoblotted.

**Figure 5.** Parameters of insulin sensitivity determined during the euglycemic hyperinsulinemic clamp performed in *Psammomys obesus*. Glucose infusion rate (GIR), glucose disappearance rate (Rd), and glucose production rate (EGP) are illustrated in A, B, and C, respectively. Data represent mean±SE for n=4 per group. *P<0.05 vs groups A and B. **P<0.05 vs group A.
model of insulin resistance. Thus, our results suggest that increased MTP confers an accelerated production of TG-rich, apoB-containing lipoproteins in the liver of Psammomys obesus affected with insulin resistance and diabetes. In fact, FFA mobilization from the adipose tissue might attenuate insulin signaling, exacerbate insulin resistance, and upregulate MTP expression via the insulin response element in the promoter region of the MTP gene. In addition, FFA influx in the liver can increase TG intracellular availability and provide ample lipid substrate for MTP activity and VLDL assembly. Therefore, increased FFA flux might be the driving force for enhanced MTP activity and VLDL overproduction.

Changes in VLDL-TG production can be due to changes in VLDL-TG production through VLDL particles, the number of VLDL particles, or both. Because 1 apoB molecule is secreted per VLDL particle, VLDL-apoB production provides an assessment of the number of VLDL particles secreted. Once the ratios of apoB to TG were calculated in our studies, they displayed higher values in groups B (2.44) and C (2.56) than in group A (1.69). Therefore, our data are consistent with an actual increase in the secretion of VLDL particles and not simply an elevation in the TG content of VLDL. These results suggest that insulin resistance and diabetic conditions modulate hepatic VLDL-TG and VLDL-apoB secretion rates by promoting the assembly of an elevated number of VLDL vehicles.

Because apoB lipidation plays an important role in regulating the assembly and secretion of apoB-containing lipoproteins, we then decided to define hepatic lipid status. Our data did indeed substantiate this hypothesis in view of high levels of TG, cholesterol ester, and phospholipid measured in liver homogenates on the 1 hand and the reduced proteasome degradation of apoB on the other hand in insulin-resistant and diabetic animals. Abundant FFAs might act as precursors of these lipids, which are capable of stabilizing the hydrophobic apoB in the lumen of the rough endoplasmic reticulum as well as stimulating VLDL assembly. Additionally, recent observations have proposed a link between hepatic MTP gene expression levels and the sustained influx of fatty acids into the liver in obese and hypertriacylglyceremic rats. Overall, excess FFAs originating from lipogenesis and extrahepatic tissues could conceivably enhance hepatic TG deposition, VLDL lipid-component building, and VLDL output, thereby contributing to hyperlipidemia under pathophysiologic states, including insulin resistance and diabetes. Compatible with this assumption are our results documenting an increased activity of MGAT and DGAT in Psammomys obesus with insulin resistance and diabetes. MGAT and DGAT are major control points for the regulation of TG synthesis by channeling FA coenzyme A toward specific metabolic fates. Recently, DGAT-deficient mice have been found to be resistant to obesity and insulin resistance.

Insulin resistance has been defined as a condition of low insulin sensitivity. This is well documented in our study, given the inability of groups B and C to suppress hepatic glucose production (gluconeogenesis) and enhance muscle glucose uptake. Additionally, increased glucose infusion rate and $R_g$ values characterized group B. Obviously, most of these processes indicate that group C is more insulin resistant than group B, confirming the failure of massive pancreatic insulin secretion to achieve glycemic control as well as the severe postreceptor defect exemplified by the overexpression of protein kinase Cε and diminished expression of Akt and GLUT4. However, despite the distinct degree of insulin resistance characterizing groups B and C, no significant differences were noted in plasma FFA levels, hepatic apoB synthesis, MTP activity, and esterifying enzymes (MGAT, DGAT). We can therefore conclude that insulin resistance alone is sufficient to induce substantial changes in lipid synthesis and apoB biogenesis in Psammomys obesus. Apparently, higher insulin resistance magnitude intensity, accompanied by hyperglycemia, is necessary to provoke increased aberrations in plasma TG, total cholesterol, and VLDL, as observed in group C.

In conclusion, the present study stresses the relation between insulin resistance/diabetes and hepatic, TG-rich, lipoprotein overproduction. Increased availability of lipids and enhanced MTP activity divert apoB from proteasomal degradation toward the secretory pathway, thus upregulating VLDL assembly and release from the liver. These in vitro results, together with evidence from recent literature, mainly indicate that heptatically derived apoB-containing lipoproteins might contribute toward the development of hypertriacylglycerolemia and metabolic dyslipidemia typical of insulin resistance and diabetes.

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**FIGURE I**

Plasma Glucose (mmol/L) vs. Body Weight (g)

- **Plasma Glucose (mmol/L):**
  - A: 2.5
  - B: 7.5
  - C: 12.5

- **Body Weight (g):**
  - A: 500
  - B: 1000
  - C: 1500

**Graphs:**

- Graph 1: Plasma Glucose (mmol/L) vs. A, B, C.
- Graph 2: Plasma Insulin (pmol/L) vs. A, B, C.
- Graph 3: Body Weight (g) vs. A, B, C.
FIGURE III

TG transfer activity (% / \( \mu g \) protein)

A  B  C

a  b