Changes of Liver Glucose Metabolism in C57BL/6 Mice Transgenic for Human Apolipoprotein ApoCIII

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Abstract: Mice overexpressing the human apolipoprotein apo CIII are a model of dyslipidemia. They become hypertriglyceridemic, hypercholesterolemic and have high blood levels of free fatty acids. Blood glucose is normal, but as the liver integrates lipid and carbohydrate metabolism, conditions of high inter-tissue circulation of energy substrates, such as fasting, may reveal hepatic alterations of glucose metabolism in these (CIII) mice. This hypothesis was explored by in situ liver perfusion in this investigation. The NTG (non-transgenic) animals showed liver and muscle glycogen content changes compatible with the fed or fasted state. In contrast, glycogen in group CIII was much lower in the fed state. The liver glucose release in group CIII after overnight fasting and adrenaline-stimulated was lower than in group NTG. Total glucose production under gluconeogenic conditions was not different between groups NTG and CIII, but glucose production from alanine was decreased in group CIII. Therefore, dyslipidemia caused by overexpression of apoCIII in mice alters the liver glucose metabolism, particularly compromising glycogen synthesis and degradation. This profile might have adverse outcomes during metabolic challenges that are more severe than fasting.

Key words: Apolipoprotein CIII, mice, liver, glucose metabolism.

1. Introduction

Dyslipidemias, together with impaired blood glucose balance, are among the major causes of development of atherosclerosis and the ensuing worldwide morbidity and mortality of patients. One of the most common agents of insulin resistance in type 2 diabetes mellitus is dyslipidemia linked to high blood levels of TGs (triglycerides) and FFAs (free fatty acids) [1-3]. Hypertriglyceridemia can be triggered by factors such as diet, alcoholism, medications, hormonal therapy and genetic propensity [4, 5].

Since 1980, several models expressing (transgenic) or lacking (knockout) genes that change the profile of blood lipoproteins were devised and made it possible to understand some of the complex issues of dyslipidemias. Among such models, Ito et al. [6] developed a lineage of transgenic mice overexpressing the gene for the apoCIII (apolipoprotein CIII). This is an important modulator of the liver turnover of triglycerides [7, 8]. It is an 8.8 kDa glycoprotein from the TG-rich lipoproteins, synthesized mostly in the liver and in lesser amounts in the intestine [8, 9]. Its overexpression can cause hypertriglyceridemia especially by delaying the removal of TGs from lipoproteins, decreasing the affinity of TG-rich lipoproteins for the LPL (lipoprotein lipase) [10] and decreasing the uptake of the VLDL (very-low density lipoprotein) and remaining chylomicrons, thus impairing their catabolism and preventing the reuptake of the TG-rich lipoproteins [11, 12]. Therefore, mice overexpressing this gene become hypertriglyceridemic, hypercholesterolemic and with high blood levels of FFAs [13]. On the other hand, the absence (knockout) or deficiency of the gene coding for apoCIII may lead
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Paradoxically, these studies show that the apoCIII mice, despite being hypertriglyceridemic, have normal blood glucose. One possible cause could be the high basal metabolic rate and mitochondrial activity through activation of the UCP (uncoupling protein) complex in this model [21, 22].

The liver entwines carbohydrate and lipid metabolism to a degree unparalleled by other tissues. Therefore, although mice transgenic for apoCIII have normal blood glucose, they may display liver alterations on glucose metabolism that may become relevant in situations of higher inter-tissue circulation of energy compounds, such as fasting. To explore this possibility, this work investigated the impact of the overexpression of apoCIII on liver glucose metabolism through the technique of in situ liver perfusion.

2. Method and Materials

The colony of C57BL6 mice transgenic for human apoCIII was established and reproduced according to international rules. They were kept in a ventilated rack, isolated from non-transgenic mice, at a local animal house, under light/dark cycles of 12/12 h, temperature of 23 ± 1 ºC and free supply of balanced rodent chow and water.

The pairings were made in heterozygosis to keep 50% of the colony as non-transgenic controls. At the age of 60 days, the mice from the resulting litters were genotyped individually and the genealogic tree carefully recorded in a log book. Genotyping consisted in dosing plasma triglycerides after overnight (12 h) fasting. Mice having triglyceridemia higher than 300 mg/dL were considered transgenic (CIII) and those below 100 mg/dL were considered NTG (non-transgenic), or controls [23].

For the experiments, male mice aging 4-6 months and weighting 25-25 g, non-transgenic (group NTG, n = 50) and transgenic for human apolipoprotein CIII (group CIII, n = 50), were used. Distinct animals of both groups, either fed or fasted overnight (14 h), were
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used for liver perfusion, biochemical assays of plasma and determination of muscle and liver glycogen content. All the procedures were approved by the Internal Commission on Biosecurity (CTNbio 819/2013) and Ethics Commission on the Use of Animals (CEAE 020/2013).

2.1 Biochemical Plasma Determinations

The animals were weighed, and blood samples were collected to assess blood glucose (glucose meter and test strips Optium® Xceed; Abbott, São Paulo, Brazil), triglycerides and cholesterol (commercial kits Labtest®; GoldAnalisa, Belo Horizonte, Brazil), and free fatty acids (Wako Chemicals; Richmond, VA, USA). The specifications of the suppliers were followed for the determinations.

2.2 Liver and Muscle Glycogen Content

The animals were euthanized through deep anesthesia (thiopental 120 mg/kg body weight, intraperitoneal). The liver and gastrocnemius were removed, weighed and immersed in liquid nitrogen. Perchloric acid (0.6 N) was added and the material was homogenized and centrifuged (10 min at 6,000 rpm). Aliquots of the supernatant (100 µL) were used to determine the free glucose content of the samples. Amyloglucosidase (50 µL), potassium bicarbonate (1 M, 50 µL) and sodium acetate (250 mM, 960 µL) were added to another aliquot (100 µL) of the supernatant. This solution was incubated at 40 °C in water bath under agitation for two hours, then the reaction was stopped with perchloric acid (0.6 M, 500 µL). After centrifugation (10 min at 6,000 rpm), the supernatant was used to determine the total glucose content. Glucose was determined through enzymatic-colorimetric method (commercial kit Labtest®). Glycogen-derived glucose was calculated and expressed as mmol glucose/g liver [24, 25].

2.3 In situ Liver Perfusion

The mice were anesthetized with thiopental (40 mg/kg body weight, intraperitoneal), the abdominal cavity was opened, and the portal and inferior cava veins cannulated as described elsewhere [26]. The liver was perfused with Krebs-Henseleit-Bicarbonate (KH) buffer, pH 7.4, saturated with O2/CO2 (95%/5%) and warmed at 37 °C. Euthanasia occurred through hypovolemic chock after blood drainage. After 30 min of stabilization (KH perfusion only), the effluent fluid was collected from the inferior cava vein each five min.

The liver gluconeogenic capacity was assessed in mice fasted overnight. After stabilization, the liver was perfused for 60 min with KH only (basal perfusion). Then, a gluconeogenic precursor dissolved in KH was perfused for another 60 min (stimulated perfusion). The following gluconeogenic precursors were used, separately, in supra-physiological concentrations (5 mM): glycerol, lactate, alanine and glutamine. The products determined in the effluent fluid, depending on the precursor employed, were pyruvate and lactate (through enzymatic methods) [26]; glucose, urea and ammonia (through enzymatic-colorimetric methods using commercial kits Labtest®). The NADH/NAD+ ratio was calculated from the data of pyruvate and lactate.

In fed animals, the liver was perfused with KH for 30 min after stabilization (basal perfusion) and then for another 30 min with KH plus adrenaline 1 µM (stimulated perfusion). The concentrations of glucose, lactate and pyruvate in the effluent fluid were determined.

The data of the perfusion experiments are presented as area under curve (AUC, in µmol/g liver) calculated from the dosages. Fig. 1 illustrates this relation for animals fasted overnight and perfused with lactate 5 mM. Glucose production (in µmol/min/g liver) is shown during basal (0-60 min) and stimulated (60-120 min) in situ perfusion with lactate 5 mM. The AUCs of each period and each group were calculated from the perfusion curves, and the values (in µmol/g liver) are shown at the insets in the graph.
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Fig. 1 Demonstrative experiment of liver glucose production, in overnight-fasted mice of groups NTG and CIII.

2.4 Statistical Analysis

The results were shown as mean ± SD (standard deviation) and compared through test \( t \) of Student using Prism® 5.0 (GraphPad; San Diego, CA, USA).

3. Results and Analysis

Table 1 shows that there was no difference \( (p > 0.05) \) between groups NTG and CIII, either fed or overnight fasted, in body weight and blood glucose. Group CIII had plasma values of TGs (triglycerides), CHOL (cholesterol), and FFAs (free fatty acids) significantly higher than group NTG (Table 1).

Fig. 2 brings the liver and skeletal muscle glycogen content. In the fed state, glycogen content of group CIII was lower than in group NTG both in liver and muscle (Fig. 2A). After overnight fasting, the groups did not differ in their liver glycogen content \( (p > 0.05, \) Fig. 2B), but as for the muscle glycogen group CIII had higher content than group NTG, of about 15%. The values in both tissues were higher in group NTG in the fed state than after fasting; in group CIII, however, the glycogen content remained below 1 mmol glucose/g liver regardless of the condition (fed or fasted).

Liver glucose, lactate and pyruvate release during basal and adrenaline-stimulated perfusion is shown in Table 2. Basal lactate release was lower in group CIII, while those of glucose and pyruvate did not differ from group NTG \( (p > 0.05) \). Adrenaline increased glucose and pyruvate release in group NTG, but decreased that of lactate, compared with the basal perfusion. In group CIII, glucose and lactate release were lower during stimulated perfusion compared with basal, while that of pyruvate increased. In this way, glucose and lactate release in group CIII were lower than those of group NTG during adrenaline perfusion.

As indicated in Fig. 3A, the liver glucose release after 14 h of fasting was about 50% lower in group CIII compared with group NTG. The NADH/NAD⁺ ratio of group CIII was 10 times higher than in group NTG (Fig. 3B) during basal perfusion.

Table 3 illustrates the liver glucose, pyruvate, lactate, urea and ammonia production during the stimulated perfusion with the gluconeogenic precursors glycerol, lactate, alanine and glutamine. Glucose production from glycerol, lactate or glutamine was similar between the groups \( (p > 0.05) \), as well as the production...
Table 1  Body weight and plasma values in fed and overnight-fasted mice of groups NTG and CIII.

<table>
<thead>
<tr>
<th></th>
<th>NTG (n = 5-12)</th>
<th>CIII (n = 5-12)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g, fed)</td>
<td>26.7 ± 0.60</td>
<td>25.5 ± 0.30</td>
</tr>
<tr>
<td>Body weight (g, fasted)</td>
<td>24.2 ± 0.37</td>
<td>23.8 ± 0.63</td>
</tr>
<tr>
<td>Blood glucose (mg/dL, fed)</td>
<td>130.2 ± 4.10</td>
<td>141.3 ± 7.30</td>
</tr>
<tr>
<td>Blood glucose (mg/dL, fasted)</td>
<td>78.4 ± 2.08</td>
<td>83.3 ± 2.13</td>
</tr>
<tr>
<td>TG (mg/dL, fed)</td>
<td>77.3 ± 4.3*</td>
<td>581.4 ± 27.5*</td>
</tr>
<tr>
<td>TG (mg/dL, fasted)</td>
<td>61.1 ± 4.0*</td>
<td>371.1 ± 19.0*</td>
</tr>
<tr>
<td>CHOL (mg/dL, fasted)</td>
<td>54.6 ± 5.9*</td>
<td>99.0 ± 10.8*</td>
</tr>
<tr>
<td>FFA (mEq/L, fasted)</td>
<td>0.028 ± 0.01*</td>
<td>0.086 ± 0.01*</td>
</tr>
</tbody>
</table>

TG: triglycerides; CHOL: cholesterol; FFA: free fatty acids.
Data are shown as mean ± SD; *p < 0.05 NTG vs. CIII.

Fig. 2  Liver and muscle glycogen content of mice of groups NTG and CIII, in the fed (A) and fasted (B) state.
Data are shown as mean ± SD; n = 6/group.
*p < 0.05 NTG vs. CIII.

Table 2  AUC of liver glucose, lactate and pyruvate release of fed mice of groups NTG and CIII during basal and adrenaline-stimulated perfusion.

<table>
<thead>
<tr>
<th>Product</th>
<th>Basal release (µmol/g liver)</th>
<th>Stimulated release (µmol/g liver)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NTG</td>
<td>CIII</td>
</tr>
<tr>
<td></td>
<td>NTG</td>
<td>CIII</td>
</tr>
<tr>
<td>Glucose</td>
<td>43.53 ± 5.83#</td>
<td>44.61 ± 4.38#</td>
</tr>
<tr>
<td>Lactate</td>
<td>23.92 ± 2.99*#</td>
<td>15.20 ± 3.43*#</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>0.04 ± 0.02#</td>
<td>0.05 ± 0.01 #</td>
</tr>
</tbody>
</table>

Data are shown as mean ± SD; n = 5/group; *p < 0.05 NTG vs. CIII; #p < 0.05 basal vs. stimulated perfusion of the group.

Fig. 3 AUC of basal glucose release (A) and NADH/NAD⁺ ratio (B) in overnight-fasted mice of groups NTG and CIII.
Data are shown as mean ± SD; n = 6/group; *p < 0.05 NTG vs. CIII.
Table 3  AUC of liver glucose, pyruvate, lactate, urea and ammonia production in the presence of gluconeogenic precursors in overnight-fasted mice of groups NTG and CIII.

<table>
<thead>
<tr>
<th>Precursor (5 mM)</th>
<th>Product (µmol/g liver)</th>
<th>NTG</th>
<th>CIII</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycerol</td>
<td>Glucose</td>
<td>10.4 ± 0.3</td>
<td>10.6 ± 0.5</td>
</tr>
<tr>
<td>Lactate</td>
<td>Glucose</td>
<td>15.0 ± 1.2</td>
<td>15.9 ± 0.8</td>
</tr>
<tr>
<td></td>
<td>Pyruvate</td>
<td>16.2 ± 1.5*</td>
<td>3.2 ± 0.3*</td>
</tr>
<tr>
<td>Alanine</td>
<td>Glucose</td>
<td>2.7 ± 0.4*</td>
<td>1.5 ± 0.2*</td>
</tr>
<tr>
<td></td>
<td>Pyruvate</td>
<td>2.7 ± 0.4*</td>
<td>0.2 ± 0.1*</td>
</tr>
<tr>
<td></td>
<td>Lactate</td>
<td>5.4 ± 0.6*</td>
<td>1.2 ± 0.4*</td>
</tr>
<tr>
<td></td>
<td>Urea</td>
<td>23.7 ± 1.3</td>
<td>25.8 ± 0.9</td>
</tr>
<tr>
<td></td>
<td>Ammonia</td>
<td>48.2 ± 0.1*</td>
<td>60.3 ± 4.1*</td>
</tr>
<tr>
<td>Glutamine</td>
<td>Glucose</td>
<td>1.1 ± 0.2</td>
<td>1.9 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>Pyruvate</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Lactate</td>
<td>1.6 ± 0.3*</td>
<td>0.4 ± 0.02*</td>
</tr>
<tr>
<td></td>
<td>Urea</td>
<td>74.2 ± 8.2</td>
<td>76.7 ± 7.9</td>
</tr>
<tr>
<td></td>
<td>Ammonia</td>
<td>88.3 ± 8.2</td>
<td>74.0 ± 5.3</td>
</tr>
</tbody>
</table>

Data are shown as mean ± SD; n = 6/group. ND: not detected; *p < 0.05 NTG vs. CIII.

Fig. 4  AUC of the NADH/NAD⁺ ratio in the presence of alanine in overnight-fasted mice of groups NTG and CIII.

Data are shown as mean ± SD; n = 6/group; *p < 0.05 NTG vs. CIII.

of urea and ammonia from glutamine (p > 0.05).

As for alanine, however, lower glucose and higher ammonia productions were recorded in group CIII compared with group NTG. The production of pyruvate and lactate in the presence of gluconeogenic precursors was lower in group CIII than in group NTG, except for glutamine, in which pyruvate was not detected in either group.

Fig. 4 shows that, in the transgenic (CIII) mice, the NADH/NAD⁺ ratio in the presence of alanine was increased by about 40% relative to group NTG.

4. Discussion

Dyslipidemias in humans are common components of metabolic syndromes and are associated with other systemic changes [27], so that the study of their direct effect on carbohydrate metabolism becomes hampered. Based on this, the study of the effect of the altered lipid profile on the liver glucose metabolism in mice overexpressing apoCIII may determine whether there
is a direct relationship between them.

The dyslipidemia of the CIII mice of this investigation was testified by the high levels of TGs, CHOL and FFAs. On the other hand, there was no difference of body weight and blood glucose between the NTG and CIII groups. Other studies also demonstrated that NTG and CIII mice have similar body weight and composition, fasting blood glucose, liver weight, locomotor activity and food ingestion [3, 23]. Increased body weight and adiposity in these mice are seen when they are fed a high-fat diet [28].

The NTG animals showed liver and muscle glycogen content changes compatible with the feeding condition (that is, higher when fed and lower when overnight fasted). In contrast, the liver and muscle glycogen contents of group CIII were much lower than those of group NTG in the fed state; at both moments (fed and fasted), the glycogen content of both tissues in group CIII was close to that of the fasted NTG mice, indicating that the fed state did not favor the replenishment of this polymer, as it happens in normal (NTG) animals. Even more, the basal liver glucose release in group CIII after overnight fasting was only 50% that of group NTG. As the liver glycogen content after fasting was similar between group NTG and CIII, this discrepancy indicates resistance or inability of the liver in releasing glucose to the perfusion fluid. It must be noted that the perfusion fluid lacks glucose and, during the basal perfusion, it does not contain gluconeogenic precursors, both conditions favoring the release of the endogenous glucose by the liver.

One possible explanation is that group CIII has dyslipidemic features (hypertriglyceridemia, hypercholesterolemia and high FFA levels) [23] and, according to the glucose-FFA cycle proposed by Randle [15], the increased oxidation of FFA by the peripheral tissues could decrease the need of replenishing blood glucose by the liver. The favored liver beta-oxidation in group CIII, for instance, could be inferred by the high NADH/NAD+ ratio calculated for the basal perfusion after overnight fasting.

Generically speaking, the effects of the FFAs on peripheral glucose use are manifest in at least four ways: (1) desensitization of glucose transporters; (2) inhibition of glucose phosphorylation by hexokinase; (3) inhibition of phosphofructokinase by citrate build-up; and (4) inhibition of pyruvate dehydrogenase, thus reducing glucose oxidation [29].

Some in vitro studies show evidence that the FFAs stimulate liver gluconeogenesis. The proposed mechanism includes the increased production of ATP and NADH and the activation of pyruvate carboxylase by acetyl-CoA, generated through beta-oxidation of the FFAs [30]. Other mechanisms that could explain the favoring of liver gluconeogenesis by FFAs would be the increased intracellular content of lipid metabolites and activation of protein kinase C-ε (and potentially other serine-kinases), leading to diminishment of the kinase activity of the insulin receptor and resulting in lower tyrosine phosphorylation of IRS, lower IRS-associated PI3-kinase activity and lower Akt activity. These interventions of the lipids on insulin signaling in the liver may ultimately lead to decreased glycogen synthesis and lower phosphorylation of FOXO, thus increasing the transcription rate of gluconeogenic enzymes (phosphoenolpyruvate carboxy kinase, glucose-6-phosphatase) [31-33]. The negative interference of the FFAs on insulin signaling could also explain the low levels of liver glycogen in group CIII even in the fed state. Under normal circumstances, glycogen synthesis is favored upon feeding by insulin action [34], an effect visible both in the liver and muscle of NTG animals.

In this investigation, the gluconeogenic capacity of the liver from different precursors was explored by in situ liver perfusion. On average, total glucose production was not different between groups NTG and CIII (29.2 vs. 29.9 µmol/g liver, respectively) and, as expected, glycerol and lactate were the precursors that increased most the liver glucose output [35]. Only glucose production from alanine was lower in group CIII, which can be the result of the larger ammonia
production, which is inhibitory to gluconeogenesis [36], in the presence of this precursor. Therefore, the suggestion that gluconeogenesis is stimulated by dyslipidemia, suggested from in vitro studies [30], is not supported by the results obtained in the perfused liver.

In the presence of all substrates, pyruvate production was lower in group CIII (Table 3). The high beta-oxidation of fatty acids by the liver of the CIII mice seems to be draining pyruvate to anaplerotic reactions, so that acetyl-CoA continues to be oxidized as it is being produced. Lactate does not seem to be the fate of pyruvate, because the concentrations of this compound were lower in group CIII (Table 3) despite the cytosolic redox state (that is, the NADH/NAD⁺ ratio) favoring lactate dehydrogenase activity towards pyruvate reduction to lactate. Pyruvate oxidation by PDH is also unlikely, because this enzyme is inhibited both by the cell redox state and acetyl-CoA, a prevailing condition in the liver of the CIII animals. The conversion of pyruvate to oxaloacetate by pyruvate carboxylase (which is stimulated by acetyl-CoA) is the probable route, and this could explain the lower conversion of alanine to glucose in group CIII, as pyruvate is the direct product of alanine deamination. The larger release of ammonia from alanine in group CIII is indicative of the predominance of this deamination.

The liver perfusion has the premise of isolating the liver while supplying it with substrates in a fluid free of glucose, lipids and hormones. Under these conditions, the basal glucose release reflects the endogenous glucose, that is, glucose previously stored in hepatocytes. In the fed animals, this basal release was high (almost four times higher than in fasted NTG animals and seven times higher in fasted CIII mice), which is indicative of glycogen stores in the liver; but adrenaline-stimulated glycogenolysis, which increased glucose release by 1.5 times in group NTG, did not increase it by more than 0.5 times in group CIII. Liver resistance or inability to respond to this glycogenolytic hormone in group CIII can be supposed. The low glycogen store of group CIII even after feeding may have limited adrenaline action, but the interference of the hyperlipidemic condition on adrenaline signaling pathways in the liver cannot be discarded.

The results of group CIII show: (1) unaltered post-prandial and fasting glycemia; (2) liver and muscle glycogen content typical of fasting, even in the fed state; (3) preservation of liver gluconeogenic and ureogenic capacity; and (4) decreased adrenaline-stimulated glycogenolysis. Dyslipidemia caused by overexpression of apoCIII in mice alters the liver glucose metabolism, particularly compromising glycogen synthesis and degradation. This profile does not have immediate consequences for systemic glucose homeostasis—which is kept normal in the CIII animals either fed or fasted for 14 hours—but points to a liver condition that might have adverse outcomes during metabolic challenges more severe than fasting, such as high-intensity exercise. During such challenges, the liver might be unable to keep glucose homeostasis, and a hypoglycemic episode could take place.

5. Conclusions

The reduced liver glycogen content and glycogenolytic response to adrenaline observed in the apoCIII transgenic mice did not change glucose homeostasis during fed or fasted states. However, liver glucose metabolism during instances of higher metabolic demand, such as high-intensity or incremental exercise, are worth exploring, as they may reveal that the dyslipidemic profile of this model could impair whole-body glucose homeostasis.

Acknowledgments

The authors are thankful to the technical staff of the Laboratory of Physiology for their assistance and Fundação de Apoio ao Desenvolvimento Científico for supporting this research.

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