Rat Hepatocyte Glucose Metabolism Is Affected by Caloric Restriction but not by Litter Size Reduction

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Abstract: This work aimed at investigating whether the liver glucose metabolism could have a role in any change of glucose homeostasis that might exist in the reduced-litter rat at the age of 60 days. Additionally, post-weaning caloric restriction and its hepatic effects were explored, as this intervention is reported as a useful measure against obesity and its related disturbances. The animals were raised in litters of nine (control, CG) or three pups during lactation. These small-litter pups were FG (fed freely) or subjected to 30% RG (caloric restriction) after weaning until 60 days of age. The increased adiposity induced by lactational overfeeding was not reversed by caloric restriction. Hepatocyte glucose metabolism and glucose tolerance test were not affected by litter size, but caloric restriction increased liver basal glucose release, diminished gluconeogenesis and retarded the glycemic decay during the insulin tolerance test. Liver glucose metabolism of young adult rats was not affected by lactational overfeeding. Up to this age, however, moderate caloric restriction had a potent influence that might compromise whole-body glucose homeostasis and prompt to insulin resistance.

Key words: Small litter, caloric restriction, glucose metabolism, rat, liver.

1. Introduction

Obesity is a chronic condition usually linked to a positive energy balance, where genetic and environmental factors are involved. It is related to the appearance and aggravation of several metabolic and cardiovascular disorders. As the post-natal nutritional environment can contribute significantly to obesity in adult life [1-3], excess weight gain during childhood is currently under serious consideration. Research demonstrates that adiposity during infancy can determine the patterns of body mass composition in adulthood and consequently contribute to disease, a phenomenon known as metabolic programming [1, 2, 4]. The mechanisms are not completely clear, but in rats they involve changes in the hypothalamic circuits controlling appetite and energy expenditure, which are established between the end of gestation and weaning [5-7].

Recently, caloric restriction at controlled and moderate levels (reduction of 10-30% of the free ingestion) has been put forward as prophylactic measure against metabolic and cardiovascular disturbances, overweight, diabetes and even cancer. In rodents and primates, it is reported that caloric restriction decreases abdominal adiposity, improves blood glucose homeostasis and the lipid profile [8-13].

In rats, litter size reduction during lactation is a classical model of overweight/obesity and increased adiposity in later life [3, 7, 14]. It is based on the modification of the post-natal nutritional environment (that is, change in the number of pups per dam during pregnancy) to produce an increase in the number of pups born to each dam and thereby to increase the total energy intake of the dam during lactation.
lactation) as a manner of programming the central controls of food intake and energy balance, resulting in the obese phenotype of the adult animal [2, 15-19].

Glycemic control is extremely important for the physiologic homeostasis and the liver plays an essential role in it, both because of its autorregulatory properties and because it is a central target of the neural and hormonal agents of glucose homeostasis [20-23]. Although changes in glycemic control are frequently observed in the reduced-litter rodent model of obesity, the role of liver glucose metabolism remains largely unexplored, and this was the purpose of this work. Additionally, it was tested if caloric restriction since weaning would restore any alterations programmed by the reduced litter.

2. Method and Materials

Wistar rats were used. The dams and their litters were kept at the animal house under regular light/dark cycles (12 h light/12 h dark) and controlled temperature (22 ± 2 °C). The handling, treatment and experiments were approved by the CEUA 3767080715 (Ethics Commission on the Use of Animals of the Institution).

The newborn litters were arranged so that each dam had three or nine pups, preferably males. Female pups were kept only to complete litter size during lactation (until 21 days of age). The dams had free access to water and standard rodent chow during gestation and lactation.

After weaning, the male pups were placed in plastic boxes in groups of three and the following experimental groups were established:

CG: rats from nine-pups litters that were given free access to water and chow after weaning;

FG: rats from three-pups litters that were given free access to water and chow after weaning;

RG: rats from three-pups litters that were given free access to water after weaning; the supply of chow was reduced by 30% relative to the ingestion of age-matched FG rats, corrected for body weight.

The experimental procedures were carried out when the rats were 60 days old after overnight fasting (approx. 14 h). Body weight and nasoanal length were recorded and used to calculate the body mass index (BMI, in g/cm²). Euthanasia was carried out through ip. injection of thiopental (120 mg/kg associated with lidocaine 5 mg/kg). Subcutaneous and visceral (retroperitoneal, epidydymal and mesenteric) fats were removed and weighed.

2.1 In vivo Glucose Assessments

For the oGTT (oral glucose tolerance test), the overnight-fasted rats were given glucose (1.5 g/kg) through gavage and blood samples were collected from a caudal incision at times 0, 15, 30, 60, 90 and 120 min, time 0 min being immediately before glucose administration. The AUC for the oGTT, representing the integrated change of glycemia during the test, was calculated taking the initial (time 0 min) glycemia of the animal as baseline.

The rats were given an ip. injection of regular insulin (1 U/kg, Novolin®, Denmark) for the ITT (insulin tolerance test). Blood samples were collected through a caudal incision at times 0, 5, 10, 15, 20, 25, 30 and 60 min, time 0 min being immediately before insulin injection. The index of glycemia decay (kITT, %/min) was calculated for the first 30 min of the ITT.

For both tests, glycemia was determined with test strips and glucometer. When the blood collections were finished, the animals were immediately returned to their cages and fed. At least five days elapsed between oGTT and ITT.

2.2 In Vitro Glucose Assessments

The rats were anesthetized with thiopental (40 mg/kg ip., associated with lidocaine 5 mg/kg), placed in surgical table and the liver exposed through laparotomy. A canulla was inserted into the portal vein and another into the inferior cava vein. The surgical details are described elsewhere [24].
perfusion fluid consisted of KH (Krebs-Henseleit) buffer (pH 7.4), heated to 37 °C and saturated with carbogenic mixture (O₂ 95%/CO₂ 5%). The liver was perfused for 15 min with calcium-free KH and then for 8-10 min with KH containing collagenase (700 U/dL) and calcium. The liver was removed, manually fractioned, and the cells were filtered and centrifuged three times (4 °C, 530 rpm, in KH containing albumin 0.2 g/dL).

Aliquots of 10⁶ cells/mL with viability higher than 80% were incubated for one hour under constant agitation in KH-containing flasks (pH 7.4, 37 °C) saturated with carbogenic mixture. A gluconeogenic precursor (glycerol, lactate, alanine or glutamine, 5 mM) [25] was added to some of the flasks, while others remained as controls. After incubation, the supernatants were collected to determine the concentrations of glucose, lactate, pyruvate, ammonia and urea. These were calculated as μmol/10⁶ cells/h. The difference in the concentrations of these compounds between the flasks with and without gluconeogenic precursors was the amount produced by the hepatocytes. Glucose, pyruvate and lactate production without gluconeogenic precursors (basal productions) were used to calculate the rates of glycolysis and glycogenolysis [24].

2.3 Statistical Analysis

All the data were expressed as mean ± SD (standard deviation). The three experimental groups were compared through Kruskall-Wallis with Dunn’s post-test at the significance level of 5%. Comparisons within the same group were made with Mann-Whitney. The statistical analyses were carried out with Prism® 5.0 (GraphPad, USA).

3. Results and Analysis

The biometric records of the groups are shown in Table 1. Body weight, nasoanal length, BMI and visceral fat were significantly increased in the FG compared with the CG. Subcutaneous fat was marginally higher in the FG compared with group CG (p > 0.05). Although caloric restriction (group RG) brought body weight and nasoanal length to the control range, BMI, visceral and subcutaneous fats remained higher in the RG than in the CG.

The oGTT is shown in Fig. 1. Group FG had initial glycemia (time 0 min) lower than the other groups. Fifteen min after oral glucose, glycemia had risen significantly in all the groups in comparison with time 0 min, but was not different among the groups (p > 0.05) at each time point. The integrated change of glycemia during the test (AUC), however, showed a higher value for group FG. Caloric restriction restored the AUC of group RG to that of group CG.

Table 2 gives the ITT data of groups CG, FG and RG after overnight fasting. Hypoglycemia was established 15 min after insulin injection in all the groups and reached its lowest values at 60 min. Group RG tended to have higher glycemia during the first 30 min of the IIH, resulting in its lower kITT. Groups CG and FG did not differ in glycemia or kITT (p > 0.05). Basal (0 min) and final (60 min) glycemia were not different across the groups (p > 0.05).

Hepatocyte glucose production under basal conditions and with different gluconeogenic substrates is shown in Table 3. Basal glucose production from the RG hepatocytes was much higher, as well as glucose

Table 1  Biometric parameters of groups CG, FG and RG at 60 days of age.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>CG (n = 9-16)</th>
<th>FG (n = 6-12)</th>
<th>RG (n = 9-15)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>229.90 ± 12.74</td>
<td>278.00 ± 13.65*</td>
<td>235.10 ± 34.40**</td>
</tr>
<tr>
<td>Nasoanal length (cm)</td>
<td>20.91 ± 0.28</td>
<td>21.82 ± 0.25*</td>
<td>20.36 ± 1.13**</td>
</tr>
<tr>
<td>BMI (g/cm²)</td>
<td>0.53 ± 0.02</td>
<td>0.58 ± 0.03*</td>
<td>0.56 ± 0.04*</td>
</tr>
<tr>
<td>Visceral fat (g/100 g)</td>
<td>1.41 ± 0.26</td>
<td>2.03 ± 0.29*</td>
<td>1.98 ± 0.32*</td>
</tr>
<tr>
<td>Subcutaneous fat (g/100 g)</td>
<td>0.42 ± 0.14</td>
<td>0.57 ± 0.14</td>
<td>0.66 ± 0.12*</td>
</tr>
</tbody>
</table>

Data shown as mean ± SD. * p < 0.05 vs. CG; ** p < 0.05 vs. FG.
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Fig. 1  oGTT of groups CG, FG and RG at 60 days of age.
Data shown as mean ± SD. * p < 0.05 vs. CG; # p < 0.05 vs. time 0 min of the group.

Table 2  Data from ITT of Groups CG, FG and RG at 60 days of age.

<table>
<thead>
<tr>
<th>Condition</th>
<th>CG (n = 9)</th>
<th>FG (n = 6)</th>
<th>RG (n = 9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycemia at 0 min (mg/dL)</td>
<td>75.22 ± 7.55</td>
<td>79.33 ± 8.73</td>
<td>84.11 ± 8.82</td>
</tr>
<tr>
<td>Glycemia at 15 min (mg/dL)</td>
<td>49.00 ± 6.06#</td>
<td>51.40 ± 3.78#</td>
<td>60.29 ± 8.58#</td>
</tr>
<tr>
<td>Glycemia at 30 min (mg/dL)</td>
<td>30.14 ± 6.28#</td>
<td>31.20 ± 4.71#</td>
<td>40.75 ± 8.01#</td>
</tr>
<tr>
<td>Glycemia at 60 min (mg/dL)</td>
<td>21.22 ± 2.82#</td>
<td>27.40 ± 2.88#</td>
<td>24.38 ± 5.50#</td>
</tr>
<tr>
<td>kITT (%/min)</td>
<td>3.98 ± 0.68</td>
<td>4.05 ± 1.22</td>
<td>2.42 ± 0.58/*/**</td>
</tr>
</tbody>
</table>

Data shown as mean ± SD. * p < 0.05 vs. CG; ** p < 0.05 vs. FG; # p < 0.05 vs. time 0 min of the group.

Table 3  Glucose production of hepatocytes from groups CG, FG and RG.

<table>
<thead>
<tr>
<th>Condition</th>
<th>CG (n = 5-6)</th>
<th>FG (n = 5-6)</th>
<th>RG (n = 5-6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal (μmol/10^6 cells/hr)</td>
<td>0.166 ± 0.071</td>
<td>0.412 ± 0.108</td>
<td>1.585 ± 0.242*</td>
</tr>
<tr>
<td>Glycerol (μmol/10^6 cells/hr)</td>
<td>0.429 ± 0.142</td>
<td>0.379 ± 0.105</td>
<td>0.627 ± 0.072**</td>
</tr>
<tr>
<td>Lactate (μmol/10^6 cells/hr)</td>
<td>1.231 ± 0.576</td>
<td>0.633 ± 0.225</td>
<td>0.286 ± 0.124*</td>
</tr>
<tr>
<td>Alanine (μmol/10^6 cells/hr)</td>
<td>0.199 ± 0.045</td>
<td>0.651 ± 0.200</td>
<td>0.069 ± 0.009**</td>
</tr>
<tr>
<td>Glutamine (μmol/10^6 cells/hr)</td>
<td>0.614 ± 0.191</td>
<td>0.212 ± 0.133</td>
<td>0.134 ± 0.069*</td>
</tr>
</tbody>
</table>

Data shown as mean ± SD. * p < 0.05 vs. CG; ** p < 0.05 vs. FG.

production from glycerol, than in the other groups. However, glucose production from lactate, alanine and glutamine were lower than those of CG or FG. The differences in glucose production between groups CG and FG did not attain significance (p > 0.05).

The rates of glycolysis and glycogenolysis in the basal condition, calculated from the basal production of glucose, pyruvate and lactate, are shown in Table 4. There was a quite wide range of values (not shown), so that the rates of glycolysis were statistically similar in all the groups (p > 0.05). On the other hand, the rate of glycogenolysis of group RG was significantly higher than in group CG, especially because of the high basal glucose production (Table 3).

Table 5 brings ammonia and urea production by hepatocytes of groups CG, FG and RG. Basal urea production was higher in group FG, but lower in the presence of glutamine. The values of group RG were similar to those of CG (p > 0.05). Ammonia production from alanine or glutamine did not differ among the groups (p > 0.05), and so was urea production from alanine.

4. Discussion

Pre- and post-natal nutritional conditions are well accepted as determinants, or programmers, of adult life body mass and adiposity [3]. The reduced-litter rat is a suitable model to investigate the general physiological
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Table 4  Basal rates of glycolysis and glycogenolysis from groups CG, FG and RG.

<table>
<thead>
<tr>
<th>Basal rate</th>
<th>CG (n = 5-6)</th>
<th>FG (n = 5-6)</th>
<th>RG (n = 5-6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycolysis (μmol/10^6 cells/hr)</td>
<td>0.077 ± 0.003</td>
<td>0.216 ± 0.091</td>
<td>0.088 ± 0.033</td>
</tr>
<tr>
<td>Glycogenolysis (μmol/10^6 cells/hr)</td>
<td>0.207 ± 0.053</td>
<td>0.520 ± 0.129</td>
<td>1.696 ± 0.390*</td>
</tr>
</tbody>
</table>

Data shown as mean ± SD. * p < 0.05 vs. CG.

Table 5  Ammonia and urea production of hepatocytes from groups CG, FG and RG.

<table>
<thead>
<tr>
<th>Condition</th>
<th>CG (n = 5-6)</th>
<th>FG (n = 5-6)</th>
<th>RG (n = 5-6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonia production</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basal (μmol/10^6 cells/hr)</td>
<td>0.076 ± 0.035</td>
<td>0.173 ± 0.095</td>
<td>0.081 ± 0.007**</td>
</tr>
<tr>
<td>Alanine (μmol/10^6 cells/hr)</td>
<td>0.152 ± 0.036</td>
<td>0.160 ± 0.076</td>
<td>0.157 ± 0.083</td>
</tr>
<tr>
<td>Glutamine (μmol/10^6 cells/hr)</td>
<td>0.112 ± 0.025</td>
<td>0.187 ± 0.113</td>
<td>0.115 ± 0.009</td>
</tr>
<tr>
<td>Urea production</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basal (μmol/10^6 cells/hr)</td>
<td>0.135 ± 0.031</td>
<td>0.630 ± 0.064*</td>
<td>0.269 ± 0.063</td>
</tr>
<tr>
<td>Alanine (μmol/10^6 cells/hr)</td>
<td>0.767 ± 0.100</td>
<td>0.530 ± 0.154</td>
<td>0.546 ± 0.167</td>
</tr>
<tr>
<td>Glutamine (μmol/10^6 cells/hr)</td>
<td>1.221 ± 0.123</td>
<td>0.460 ± 0.113*</td>
<td>1.037 ± 0.079**</td>
</tr>
</tbody>
</table>

Data shown as mean ± SD. * p < 0.05 vs. CG; ** p < 0.05 vs. FG.

and metabolic disorders triggered by lactational overfeeding.

Changes in glucose homeostasis, or systemic glucose turnover, are frequently reported in the reduced-litter rat [18, 26]. This work aimed at investigating whether the liver glucose metabolism could have a role in any change of glucose homeostasis that might be present in the reduced-litter rat at the age of 60 days. Additionally, post-weaning caloric restriction and its hepatic effects were explored, as this intervention is reported as a useful measure against obesity and its related disturbances [8-13, 27].

Early overfeeding had a significant impact on the biometric parameters of the adult reduced-litter (FG) rats (Table 1). Body weight and length, BMI and visceral fat, were all significantly increased, as expected from other investigations [2, 3, 14, 28-30]. What was most surprising, though, was that caloric restriction since weaning did not completely reverse this status (Group RG, Table 1): visceral and subcutaneous fat, as well as BMI, remained higher than those of the CG (control group). Thus, it seems that caloric restriction soon after weaning affects body growth of the reduced-litter rat, but apparently not by immediately decreasing fat stores. Fat deposition, therefore, can be regarded as a feature programmed by litter size reduction that was not reversed by caloric restriction until young adulthood.

Glucose production by the isolated hepatocytes was not affected by litter size, as can be seen in Table 3: groups CG and FG were similar on their production of glucose, both in the basal condition and in the presence of gluconeogenic precursors. This is in marked contrast with group RG, where basal glucose production was larger while glucose production from lactate, alanine and glutamine was lower than on the other groups. This suggests that, on the reduced-litter young adult rat, liver glucose metabolism is not significantly shifted from its normal (control) status; in other words, it is not programmed by early life overfeeding. This is in accordance with data from older animals [31] and thus seems to be age-independent.

On the other hand, group RG had higher glucose production and basal rate of glycogenolysis, both of which are indicative of glycogen stores even after overnight fasting. Consistent with this idea, higher glycogen content and similar findings on liver glucose metabolism were reported in reduced-litter, calorically-restricted rats aging 90 days [31]. As rats from large litters subjected to caloric restriction after weaning also display these patterns of liver glucose
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metabolism [24, 32, 33], it can be assumed that caloric restriction had an impact on liver glucose metabolism that seems to be dependent of the prevailing nutritional condition, and not of age or litter size.

An attempt was made to correlate liver glucose metabolism with in vivo glucose handling during glucose or insulin challenges (oGTT and ITT, respectively). The AUC of oGTT was increased in group FG, but as its liver glucose metabolism was unaltered compared with group CG, it is supposed that other tissue(s) might account for the observed in vivo glycemia profile during the test. For instance, a decreased insulin action in adipocytes of rats from reduced litters is suggested [2], which would diminish the response to endogenous insulin released by oral glucose in the fat-rich FG. Although groups FG and RG had equal amounts of fat, caloric restriction might have normalized insulin action on this tissue even without changing its weight. In addition, epigenetic modifications of key genes related to the response of skeletal muscle to insulin were found in small-litter rats, which would compromise insulin action on this tissue [34].

On the other hand, liver glucose metabolism seemed more correlated with the glycemic profile during the ITT. Groups CG and FG were very similar in their kITT and liver glucose production, while group RG had higher kITT (an indicative of insulin resistance) and basal hepatocyte glucose production. In this situation, the increased liver glycogen stores of group RG (inferred from the higher basal hepatocyte glucose production) may have impaired the liver capacity of responding to the exogenous insulin by synthesizing glycogen and decreasing glycemia.

The observed changes in nitrogen release (ammonia and urea) in group FG were not seen in group RG, which was essentially equal to group CG. Therefore, hepatocyte nitrogen handling was changed by litter size reduction, but restored to normal by caloric restriction after weaning. Later in life, urea production from alanine and glutamine in RG rats actually decreases, but those animals had less fat and lower body weight even when compared with control rats and would be recycling nitrogen instead of releasing it to the incubation medium, implying that nitrogen metabolism changes in an age-dependent manner, with older animals possibly favouring transamination [31].

In summary, hepatocyte glucose metabolism was not altered by lactational overfeeding in 60-days-old rats, and could not account for the more persistently elevated glycemia during the oral glucose load. Caloric restriction, on the other hand, did change hepatocyte metabolism and caused insulin resistance, as assessed by kITT. As these findings were absent from the reduced-litter, freely-fed rats, they cannot be ascribed to early programming, but instead to the current nutritional condition. Therefore, increased adiposity was the only parameter assessed in this investigation that was programmed by lactational overfeeding, and up to 60 days of age it was not reversed by post-lactational caloric restriction.

5. Conclusions

Liver glucose metabolism of young adult rats was not subject to programming due to lactational overfeeding. Up to this age, however, moderate caloric restriction had a potent influence on liver glucose metabolism by enhancing glycogen stores and decreasing gluconeogenesis. This might impair whole-body glucose homeostasis and prompt to insulin resistance conditions that are undesirable in any nutritional intervention.

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References

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