



# Regulation of ammonia-metabolizing enzymes expression in the liver of obese rats: Differences between genetic and nutritional obesities

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**OBJECTIVE:** To determine the expression of carbamoylphosphate synthetase (CPS) and glutamine synthetase (GS) in two different models of obese rats: genetically obese rats and diet obese rats.

**SUBJECTS:** Lean and genetically obese (fa/fa) Zucker rats were used.

**DESIGN:** Lean animals (30–60 d old) were fed for 30 d with standard chow pellets or with a hypercaloric cafeteria diet. Genetically obese rats were fed with standard chow pellets.

**MEASUREMENTS:** Enzyme activity, protein (Western blot) and mRNA (Northern blot) contents of CPS and GS were measured in liver homogenates.

**RESULTS:** In genetically obese animals CPS mRNA content was higher, and GS mRNA content was lower than in control animals; CPS protein content did not change and CPS activity was lower than in control rats. Diet-obese rats had higher levels of CPS and GS mRNAs than control animals; GS protein content and activity was higher than in the control group and at the same time, CPS activity was very low.

**CONCLUSIONS:** In the genetically obese animals the expression of CPS and GS is mainly regulated at the pre-translational level, whereas in the diet obese rats there is a noticeable post-translational component. A reciprocal regulation between CPS and GS can be established at pre-translational levels, whereas at post-transcriptional levels it cannot. It can be concluded that in diet-obese animals the mechanisms involved in retaining nitrogen (low CPS activity) are modulated at the post-translational level.

**Keywords:** carbamoylphosphate synthetase; glutamine synthetase; obesity; gene expression; liver

## Introduction

The accumulation of inappropriate amounts of fat into white adipose tissue leads to the development of obesity, which affects the way nitrogen (N) is used in several ways.<sup>1,2</sup> Various models of obesity, with genetic and nutritional causes, have been used to study how this condition affects N use.<sup>1–3</sup> Higher retention of N has been reported in cafeteria-fed rats, a model of voluntary hyperphagia of fat-rich food in which animals are offered a variety of palatable foods.<sup>1</sup> The reduced excretion of N in this nutritional model of obesity is mainly due to a decrease in amino acid catabolism and urea production by the liver.<sup>4</sup> In contrast, the urea production/nitrogen excretion of genetically obese Zucker fa/fa rats seems unchanged,<sup>2</sup> and the two groups use N derived from amino acids (mainly alanine) or from ammonia in different ways.<sup>3</sup> In fa/fa rats, this situation co-exists with increased protein accumulation and

extreme fat storage.<sup>5</sup> The mechanisms that regulate N economy in these metabolic situations have not been fully defined, in spite of general agreement about the mechanism of N distribution in liver. Nitrogen derived from amino acids is converted into urea in the periportal and proximal pericentral cells; the ammonia which escapes ureagenesis in upstream areas is converted into glutamine and scavenged in the distal pericentral hepatocytes.<sup>6</sup> This hepatic complementary distribution of the rate-determining enzyme of urea synthesis, carbamoylphosphate synthetase I (EC 6.3.4.16), and glutamine synthetase (EC 6.3.1.2) is highly suggestive of a reciprocal regulation of gene expression.<sup>7</sup> Here we investigate whether this relationship persists under conditions of N-retention (nutritionally obese animals) and without N-retention (genetically obese rats).

## Materials and methods

### Animals and diets

Zucker lean (FA/?) and obese (fa/fa) adult male rats were used. They were kept in controlled light (12 h on/12 h off) and temperature (21 ± 1°C) conditions.

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All the rats had free access to both drinking water and standard chow pellets (A04 Panlab, Barcelona, Spain). On day 30 of life the lean rats were randomly divided into two groups: one group was fed with a cafeteria-diet (diet-obese group) and the other with the standard-diet (control group). Obese rats were fed with standard diet (fa/fa group). The cafeteria diet used in our laboratory has been reported elsewhere.<sup>8</sup> The main difference between both diets was that the total derivable energy from lipids was ca. 8% in the standard diet and ca. 30% in the cafeteria diet.<sup>5</sup> However, the amount of protein ingested by these animals was the same in each group,<sup>5</sup> irrespective of the significant variations registered in the total caloric intake.<sup>9</sup> Animals from these three experimental groups were killed by cervical dislocation when rats were 60 d old, and their livers were rapidly removed, frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$ .

#### RNA isolation and Northern blot analysis

Total RNA was isolated by the guanidinium thiocyanate/phenol/chloroform method<sup>10</sup> and stored at  $-80^{\circ}\text{C}$ . For Northern-blot analysis, the RNA was size-fractionated by 1% agarose-gel electrophoresis in 2.2M formaldehyde. The gels were blotted onto nitrocellulose membranes (BA-S 85, Schleicher & Schull). The RNA was irreversibly bound to the membranes by air-drying and heating at  $80^{\circ}\text{C}$  for 1 h. CPS and GS mRNAs were identified by hybridization with their respective cDNA probes.<sup>7</sup> DNA fragments were labelled with ( $\alpha$ - $^{32}\text{P}$ )dCTP by using the Multiprime labelling kit (Amersham International). The mRNA was quantified by densitometry using an EPSON-8000 scanner with GELIMAGE software (Pharmacia, Sweden).

#### Western blot analysis

Protein extracts were prepared by homogenizing the liver in 10 volumes of 0.25 M sucrose, 50 mM K-phosphate pH 7.5, 1 mM dithiothreitol, 0.1% (v/v) Triton X-100 using a Potter-Elvehjem homogenizer. The protein concentration was determined according to Lowry *et al.*<sup>11</sup> Samples containing equal amounts of total protein were loaded onto gels. After electrophoresis of the extracts on 10% (w/v) polyacrylamide gels in the presence of SDS,<sup>12</sup> the gels were blotted onto nitrocellulose membranes (BA-S 85, Schleicher & Schuell). Prestained molecular weight marker proteins

(Sigma) were transferred simultaneously to enable the antibody binding bands to be identified. To detect CPS and GS protein, the membranes were incubated with the respective antibodies.<sup>13</sup> Primary antibody binding was detected using goat anti-rabbit immunoglobulin antibodies and rabbit-peroxidase-antiperoxidase complex (Cultek, Spain). The immuno-complex formed was visualized by incubating the membranes in 50 mM Tris/HCl pH 7.6 0.5 mg/ml 3,3'-diaminobenzidine tetrahydrochloride, 0.03% (v/v)  $\text{H}_2\text{O}_2$ . The colour reaction was stopped by incubating the membranes for 1 minute in 1% (v/v) HCl. The amounts of protein were quantified by densitometry as for Northern blot.

#### Carbamoylphosphate synthetase and glutamine synthetase activities

Liver carbamoylphosphate synthetase was determined by measuring the hydroxyurea produced from carbamoyl-phosphate in the presence of N-acetylglutamate.<sup>14</sup> Glutamine synthetase activity was determined by quantifying ferric salts complexes with  $\gamma$ -glutamylhydroxamate derived from glutamine.<sup>15</sup> Protein was determined according to Lowry *et al.*<sup>11</sup>

#### Statistical analysis

Differences between groups were estimated by one-way analysis of variance (ANOVA). When the F-test was significant, the Student Newman-Keules test was used to determine differences between group means.

## Results

The initial weight of the animals was:  $69 \pm 3$  g (FA/?) and  $82 \pm 4.5$  g (fa/fa). On day 60, the control group of animals fed with the standard diet weighed  $220 \pm 6$  g, animals fed the cafeteria diet weighed  $280 \pm 12$  g and fa/fa rats weighed  $320 \pm 15$  g. Significantly higher GS activity was shown by the diet-obese group but no difference was observed between fa/fa rats and control animals (Table 1). The CPS activity (Table 2) was slightly lower in genetically obese rats; this contrasted with the low activity of the diet-obese rats, which was only 15% of that of the control group. The amount of GS protein (Table 1) was four times higher in diet-obese animals, and did not change in the fa/fa group.

**Table 1** Effect of genetic and nutritional obesity on glutamine synthetase

	Activity*	Control %	Protein**	Control %	mRNA**	Control %
Control	$0.8 \pm 0.1^a$	100	$10.0 \pm 1.0^a$	100	$28.9 \pm 1.1^a$	100
fa/fa	$0.8 \pm 0.1^a$	100	$10.1 \pm 0.9^a$	100	$12.0 \pm 2.2^b$	40
Diet obese	$1.2 \pm 0.1^b$	150	$40.1 \pm 6.0^b$	400	$59.1 \pm 2.2^c$	200

\*Values are mean  $\pm$  SEM of 6 animals and \*\* of 3–4 animals. Equivalent amounts of RNA and protein were loaded on to the gels. Statistical analysis of ANOVA and Student Newman-Keuls test: significant differences between the groups are expressed by different superscripts ( $P < 0.05$ ). \*nkat/mg protein. \*\*arbitrary units. Enzyme activity, protein and mRNA measured in control animals are regarded as 100%.

**Table 2** Effect of genetic and nutritional obesity on carbamoylphosphate synthetase

	Activity*	Control %	Protein**	Control %	mRNA**	Control %
Control	558.2 ± 10.0 <sup>a</sup>	100	220 ± 19.5 <sup>a</sup>	100	11.6 ± 1.6 <sup>a</sup>	100
fa/fa	461.4 ± 31.8 <sup>b</sup>	80	240 ± 21.1 <sup>a</sup>	110	73.3 ± 1.0 <sup>b</sup>	600
Diet obese	86.1 ± 5.5 <sup>c</sup>	15	230 ± 20.1 <sup>a</sup>	105	15.1 ± 0.9 <sup>a</sup>	130

\*Values are mean ± SEM of 6 animals and \*\* of 3–4 animals. Equivalent amounts of RNA and protein were loaded onto the gels. Statistical analysis by ANOVA and Student Newman–Keuls test: significant differences between the groups are expressed by different superscripts ( $P < 0.05$ ). \*pkat/mg of protein, \*\*arbitrary units. Enzyme activity, protein and mRNA measured in control animals are regarded as 100%.

**Table 3** Activity/protein and protein/mRNA ratios of glutamine synthetase and carbamoylphosphate synthetase

Ratios	Glutamine synthetase		Carbamoylphosphate synthetase	
	Activity/protein	Protein/mRNA	Activity/protein	Protein/mRNA
Control	100	100	100	100
fa/fa	100	250	73	17
Diet-obese	35	200	14	77

mRNA, protein and activity levels found in control animals are regarded as 100%. The values of fa/fa and diet-obese rats are presented as percentages of control values.

In the case of CPS (Table 2) there were no differences between the groups. Hepatic GS mRNA content was 2.5 times lower in fa/fa rats and double in diet-obese rats in comparison to the control group (Table 1). The amount of CPS mRNA was six times higher in fa/fa rats and did not vary in diet obese rats (Table 2). The GS mRNA levels were lower while the CPS mRNA levels were higher in genetically obese rats compared to the control values. Diet-obese rats showed a tendency to reverse this trend, since the levels of GS mRNA were higher than control values while CPS mRNA levels were maintained. The ratios of enzyme activity/protein and enzyme protein/mRNA of both enzymes are shown in Table 3. The GS activity/protein ratio was decreased significantly in the diet-obese group and the protein/mRNA ratio increased in the diet-obese ( $\times 2$ ) and fa/fa ( $\times 2.5$ ) groups. The CPS activity/protein ratio was decreased dramatically in diet-obese rats, as was the protein/mRNA ratio in fa/fa rats.

## Discussion

Hepatic nitrogen management has been shown to vary in different models of experimental obesity.<sup>3</sup> Animals fed with hypercaloric diets—such as the cafeteria diet—tend to spare nitrogen by decreasing the production of urea by the liver<sup>1</sup> as well as its excretion in the kidney.<sup>2,3</sup> Genetically obese rats do not have these nitrogen sparing patterns since urea nitrogen excretion is higher than that of rats fed with a reference diet.<sup>2,3</sup> Since both genetically obese and diet-obese rats show hyperphagia<sup>2,16</sup> we chose to investigate how an excessive energy supply affects the development of these metabolic patterns. Although there have been many studies into the induction of enzyme activities related to N disposal by increasing the protein content

of the diet,<sup>17</sup> our results would seem to indicate that the role of dietary energy content in the disposal of N is subsidiary to other hormonal or metabolic effectors. We assume that the activity, protein and mRNA levels that we measured reflect steady state conditions, because the respective diets were available to the animals for a prolonged period (30 d). Furthermore, we assayed enzyme activities under optimal conditions, so we can assume that the changes in the ratio of enzyme activity and enzyme protein content reflect structural, post-translational changes in the protein. If we accept these assumptions, the data in Tables 1–3 suggest that there is a major difference in the level at which diet and genetics affect the gene expression of CPS and GS.

Genetically obese male Zucker rats are characterized by hyperinsulinemia with peripheral insulin resistance, hypercorticism and hyposomatotropism.<sup>18–24</sup> Such an endocrinological status fits with a higher CPS expression (six fold increase in hepatic mRNA levels) and a lower GS expression (2.5 fold decrease in hepatic mRNA levels),<sup>25</sup> but the magnitude of the effects that we observed was more pronounced than has been reported for hypophysectomized or glucocorticosteroid-treated diabetic rats.<sup>26–28</sup> In animals with cafeteria-diet induced obesity, the large, reciprocal changes in CPS and GS expression that characterize the fa/fa background were not found, and the hepatic CPS mRNA content only increased a modest 1.3 times and GS mRNA twice. Diet-induced obesity is associated with hyposomatotropism<sup>29,30</sup> and hyperinsulinemia.<sup>4,31</sup> However, it should be taken into account that this hyperinsulinemia is not clearly established in this hypercaloric (mainly high fat) diets.<sup>32</sup> In any case, the comparison between genetically obese and diet-obese rats shows that it is the well-defined, but abnormal hormonal status of fa/fa rats that may be responsible for the pretranslational differences observed in CPS and GS expression.

At present, we do not know to what extent these differences are caused by changes in the rate of transcription of the respective genes or by changes in the stability of the respective mRNAs, although CPS mRNA has been stabilized by hormonal activation in cultured rat hepatocytes.<sup>33</sup> However, perhaps even more interesting are the pronounced post-transcriptional regulatory events that we observed. The most explicit difference in the protein:mRNA ratios, which reflects changes in translational efficiency and/or in protein stability, was observed in obese fa/fa rats with a six fold decrease in CPS and a 2.5 fold increase in GS. These post-transcriptional events, therefore, completely neutralized the effects observed at the mRNA level. Similar, but more modest, changes were observed in diet-obese rats. However, they also showed a six fold decline in the CPS activity:protein ratio and a three fold decline in that of GS, whereas this ratio remained unchanged in fa/fa rats. The activity:protein ratio reflects post-translational changes in the (intact) protein, as can be deduced from our results. In accordance with the flux-determining role of CPS and GS in the ornithine cycle and glutamine synthesis, respectively, these changes in Vmax reflect changes in metabolite flux.<sup>1,3</sup> When comparing the effects of our high-fat diets with those of high-protein diets it should be pointed out that the latter mostly result from changes in enzyme mass, that in turn result from changes in the rate of gene expression,<sup>17</sup> whereas the former are due to post-translational changes which cause a decrease in the molecular specific activity (activity per enzyme molecule) of the enzymes. Furthermore, the data show that CPS and GS are reciprocally regulated by factors that act at the pre-translational, that is to say probably at the transcriptional, level whereas reciprocity is not observed when post-transcriptional regulation is involved.

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