Up-regulation of liver glucose-6-phosphatase in rats fed with a P_i -deficient diet

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Because P_i deprivation markedly affects the Na/P_i co-transporter in kidney and has been related to insulin resistance and glucose intolerance, the effect of a P_i -deficient diet on the liver microsomal glucose-6-phosphatase (G6Pase) system was investigated. Rats were fed with a control diet (+ P_i) or a diet deficient in phosphate ($-P_i$) for 2 days and killed on the morning of the third day, after an overnight fast (fasted) or not (fed). Kinetic parameters of P_i transport ($t_{1/2}$ and equilibration) into liver microsomes were not changed by the different nutritional conditions. In contrast, it was found that G6Pase activity was significantly increased in the ($-P_i$) groups. This was due to an increase in the V_{max} of the enzyme, without change in the K_m for G6P. There was no correlation between liver microsomal glycogen content and

G6Pase activity, but both protein abundance and mRNA of liver 36 kDa catalytic subunit of G6Pase (p36) were increased. The mRNA of the putative G6P translocase protein (p46) was changed in parallel with that of the catalytic subunit, but the p46 immunoreactive protein was unchanged. These findings indicate that dietary P_i deficiency causes increased G6Pase activity by up-regulation of the expression of the 36 kDa-catalytic-subunit gene.

Key words: catalytic subunit, glucose intolerance, hypophosphataemia, putative glucose 6-phosphate transporter, rat liver microsomes.

INTRODUCTION

Liver glucose-6-phosphatase (G6Pase) is a multiprotein complex that resides in the endoplasmic-reticulum membrane and plays an important role in the regulation of blood glucose concentration [1]. Until now, two components have been cloned: a 36 kDa catalytic subunit (p36) [2] and more recently a 46 kDa protein (p46) [3] that has been proposed to be a glucose 6-phosphate (G6P) transporter. P36 gene expression has now been well studied. Positive effectors that increase p36 mRNA include glucose, fatty acids, cyclic AMP and glucocorticoids, whereas insulin decreases p36 mRNA [4]. Accordingly, during fasting and in diabetes, when the insulin/glucagon ratio is low, p36 mRNA, protein and activity are high [5]. Hormonal control of p46 has not yet been reported. P_i imbalance leads to a number of disorders [6,7]. Hypophosphataemia in several diseases (hypophosphataemic rickets, adult-onset hypophosphataemic osteomalacia, renal P_i leak) is associated with insulin resistance and glucose intolerance [8], but this association depends on the severity of hypophosphataemia [9]. A P_i -deficient diet causes up-regulation of the expression of the rat kidney Na/P_i -2 co-transporter [10–12], while a potential effect of P_i deprivation on the liver endoplasmic-reticulum G6Pase complex, which includes a P_i -transport system, has not been investigated.

Our results show that a P_i -deficient diet up-regulates rat liver microsomal G6Pase activity by increased expression of the p36-catalytic-subunit gene, but without significant change in the abundance of the putative G6P translocase p46 protein.

EXPERIMENTAL

Materials

$KH_2^{32}PO_4$ and $[U-^{14}C]G6P$ were from ICN Biomedicals (Montreal, QC, Canada) and $[\alpha-^{32}P]dCTP$ was from Pharmacia

Biotech (Baie d'Urfé, QC, Canada). BSA, G6P and amyloglucosidase were from Sigma (St. Louis, MO, U.S.A.). Hexokinase and G6P dehydrogenase were purchased from Boehringer (Laval, QC, Canada). Molecular markers were purchased from Bio-Rad (Mississauga, ON, Canada). The rabbit polyclonal antiserum against the recombinant G6Pase catalytic subunit (antibody against p36) was kindly given by Dr. J. Y. Chou (National Institutes of Health, Bethesda, MD, U.S.A.). The rabbit antiserum against the N-terminus of p46 (GYGYRT-VIFSSAMFGGY) was produced in our laboratory. The 5-bromo-4-chloroindol-3-yl phosphate/Nitroblue Tetrazolium (BCIP/NBT) stock solutions and the anti-rabbit IgG-alkaline phosphatase conjugate were from the Promega Co. (Madison, WI, U.S.A.). TRIZOL LS Reagent was from Gibco BRL (Burlington, ON, Canada).

Animals

Male Sprague–Dawley rats (300–350 g body weight) were used as the liver donor. Rats were fed for 48 h with either a P_i -deficient diet (no. 86128) ($-P_i$) or a control (no. 86129) (+ P_i) diet (Teklad, Madison, WI, U.S.A.) containing 0.03 % (w/w) or 1 % (w/w) P_i respectively. All other components were the same in both diets. The fasted group had food removed 18 h before being killed by decapitation, whereas the fed group had constant access to food. According to these conditions, rats were classified into four groups: 1, fed ($-P_i$); 2, fed (+ P_i); 3, fasted ($-P_i$); 4, fasted (+ P_i). There were five rats in groups 1–3 and six rats in group 4.

Preparation of rat liver microsomes and G6Pase assay

Rat liver microsomes were isolated individually as described previously [13], except that the buffer was 250 mM sucrose/50

Abbreviations used: G6P, glucose 6-phosphate; G6Pase, glucose-6-phosphatase; p36, G6Pase catalytic subunit; p46, putative G6P translocase protein; BCIP, 5-bromo-4-chloroindol-3-yl phosphate; NBT, Nitroblue Tetrazolium; EQU, tracer accumulation inside microsomes at transport equilibrium stage.

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mM Hepes/Tris, pH 7.3. G6Pase activity was assayed as described in [14], before and after detergent (0.8% Chapso) treatment.

Glycogen

Glycogen was assayed in liver microsomes with the amylo-glucosidase assay [15].

P_i transport

Zero-trans P_i transport across the microsomal membrane was measured as described previously [13].

Western-blot analysis

Rat liver microsomes (50 µg) were subjected to SDS/12%-PAGE at 100 V for about 1 h in Laemmli buffer. Then proteins were electrotransferred to a nitrocellulose membrane at 100 V for 1 h. The membrane was saturated for 30 min in 100 mM TBS (100 mM Tris/HCl/0.9% NaCl, pH 7.5), containing 10% (w/v) dried milk. Membranes were incubated in the primary antibody solution (diluted to 1:500 for anti-p36 or 1:200 for anti-p46). The membrane was washed and incubated in the alkaline phosphatase-conjugated anti-rabbit IgG solution (diluted 1:2000) for 1 h. The membrane was washed again and detected with the BCIP/NBT substrate system. The membrane was scanned and the quantification of each specific band was analysed by using a Dual Light[™] Transilluminator (Alpha Innotech Corp., San Leandro, CA, U.S.A.).

Northern-blot analysis

Total liver RNA was individually extracted with TRIZOL LS Reagent. The same amount of RNA was fractionated by 1%-agarose/formaldehyde-gel electrophoresis, then transferred to a nylon membrane by a capillary technique in 20×SSC solution. The membrane was rinsed in 2×SSC solution, dried and immobilized under UV light (1×SSC is 0.15 M NaCl/0.015 M sodium citrate). The hybridization was performed with [α -³²P]dCTP-labelled probe (full-length cDNA of p36 or p46) overnight at 68 °C. The membrane was washed properly and exposed to a film for a certain time. The intensities of the mRNA bands were determined using a densitometer (Dual Light[™] Transilluminator).

Data analysis

P_i transport kinetic analysis was done according to eqn. (1):

$$A = EQU(1 - e^{-kt}) + A_0 \quad (1)$$

in which A represents the tracer accumulation into microsomes at time t , EQU is the tracer accumulation at equilibrium, k is the rate constant and A_0 the background radioactivity.

The parameter $t_{1/2}$, the time at which 50% of a process has been completed, was calculated using eqn. (2):

$$t_{1/2} = \ln 2/k \quad (2)$$

Statistical analysis was performed according to Student's t -test. Differences were considered significant when the P value was less than 0.05.

RESULTS AND DISCUSSION

P_i transport

Renal Na/P_i co-transport is markedly up-regulated by dietary P_i deprivation [10–12], but it is not known whether liver microsomal

Table 1 Kinetic parameters for phosphate transport in microsomes: effect of fasting and of a P_i-deficient diet

P_i transport in intact microsomes was measured with tracer (10 µM) KH₂³²PO₄ for different times up to 2 min, the time at which equilibrium of uptake was reached. Results are means ± S.E.M. for each group ($n = 4-5$). $t_{1/2}$ and EQU values are not statistically different from each other respectively in the four groups.

Group of rats	$t_{1/2}$ (s)	EQU (pmol/mg of protein)
Fed (–P _i)	23 ± 1.4	8.7 ± 0.3
Fed (+P _i)	22 ± 1.3	10.0 ± 0.4
Fasted (–P _i)	24 ± 0.9	9.0 ± 1.1
Fasted (+P _i)	22 ± 1.7	10.0 ± 1.7

Figure 1 Effect of fasting and a P_i-deficient diet on microsomal G6Pase activity

G6Pase activity was measured with 0.2 mM G6P at 30 °C for 2 min. Results are mean values ± S.E.M. for each group ($n = 5-6$). Bars headed by the same letters are statistically different from each other by: ^a $P < 0.001$; ^b $P < 0.02$; ^c $P < 0.01$; ^d $P < 0.01$ (unpaired Student's t -test).

P_i transport is also affected by this diet. Liver microsomes were prepared individually from each rat in the four experimental groups. Table 1 shows that the $t_{1/2}$ of tracer KH₂³²PO₄ (10 µM) uptake into microsomes was about 23 s and did not change with the nutritional conditions (fasted or fed, + P_i or – P_i). The transport process reached an equilibrium after about 2 min, as we documented before [13], and the tracer accumulation at that time (EQU) was about 9 pmol/mg. Similarly to the $t_{1/2}$, EQU values were not statistically different for the four experimental groups. These results show that the transport rate and the P_i accessible space of liver microsomes were unaffected either by fasting or by a (–P_i) diet.

G6Pase activity

The reported association of P_i deprivation and glucose intolerance [8,9] lead us to further investigate the liver G6Pase system under our experimental conditions, since G6Pase is

Table 2 Kinetic parameters for G6P hydrolysis in microsomes: effect of fasting and of a P_i-deficient diet

The G6Pase activity of intact or detergent-treated microsomes was measured with 0.2, 0.5, 1, 2 and 5 mM G6P for 2 min at 30 °C. $V_{\max}$ and K_m were calculated by non-linear regression of Michaelis–Menten kinetics. Results are mean values $\pm$ S.E.M for each group ($n = 5-6$). Values headed by the same letters are statistically different from each other by $P < 0.02$ (unpaired Student's *t*-test).

Group of rats	$V_{\max}$ (munits/mg)		K_m (mM)	
	Intact	Detergent-treated	Intact	Detergent-treated
Fed (–P _i)	176 $\pm$ 8 ^a	330 $\pm$ 10 ^c	2.66 $\pm$ 0.14	2.15 $\pm$ 0.19
Fed (+P _i)	132 $\pm$ 4 ^a	194 $\pm$ 8 ^c	2.68 $\pm$ 0.13	2.13 $\pm$ 0.08
Fasted (–P _i)	214 $\pm$ 3 ^b	381 $\pm$ 11 ^d	2.90 $\pm$ 0.10	2.26 $\pm$ 0.15
Fasted (+P _i)	158 $\pm$ 5 ^b	321 $\pm$ 9 ^d	2.70 $\pm$ 0.11	2.35 $\pm$ 0.47

Figure 2 Comparison of microsomal glycogen content and G6Pase activity

Glycogen content was measured in microsomes as described in the Experimental section. G6Pase activity was measured at 0.2 mM G6P in intact microsomes at 30 °C for 2 min.

responsible for hepatic glucose production and the maintenance of euglycaemia. Figure 1 shows that the activity of G6Pase, measured in liver microsomes with 0.2 mM G6P before and after detergent treatment, was increased in both fed and fasted groups of (–P_i) rats compared with controls (+P_i). It can be seen that this diet-induced increase in activity was additive with the well-documented proper effect of fasting [16] as well as with that resulting from detergent treatment. The increase in G6Pase activity due to the (–P_i) diet was caused by an increase in $V_{\max}$ rather than a change in the K_m of G6Pase for G6P (Table 2). Table 2 also shows that fasting increased significantly the $V_{\max}$ of G6Pase and that the K_m of G6Pase for G6P decreased in the presence of detergent, as has been shown previously. From these results it could be deduced that the (–P_i) diet up-regulates G6Pase activity in liver.

It has been reported that glycogen-bound fatty acids regulate G6Pase activity and might be responsible for changes in this

enzyme activity in the fasting–refeeding period [17]. We thus explored the possibility that increased G6Pase activity was due to a lower liver microsomal glycogen concentration in the (–P_i) group. This was, however, not the case. As shown in Figure 2, the microsome glycogen content decreased to a large extent by fasting, but there was no obvious correlation between glycogen content and G6Pase activity.

We further verified whether up-regulation of G6Pase activity by dietary P_i deprivation was caused by changes in the protein amount of G6Pase p36 catalytic subunit and/or of p46 putative G6P transporter.

Western analysis of p36 and p46 G6Pase protein

The abundance of immunoreactive microsomal protein was measured with a polyclonal antibody against the catalytic subunit of G6Pase. The visualized band was at a position corresponding to a molecular mass of about 36 kDa (Figure 3A). The corresponding density scan results are shown in Figure 3(C). One can see that the effect of the (–P_i) diet on G6Pase activity was accompanied by a parallel increase in p36 catalytic subunit abundance and that, as for G6Pase activity, there was an additive effect of (–P_i) diet and fasting on this parameter.

The 46 kDa component of G6Pase is a putative G6P transporter, which may also be a regulator of the G6Pase catalytic subunit. It was thus possible that a (–P_i) diet has some effect on this protein, resulting in the change in G6Pase activity. An antibody against the N-terminal part of p46 was used to detect p46 protein abundance. The scan results of the specific band are shown in Figure 3(B). It is apparent that, unlike p36, the p46 protein associated with microsomes was not significantly changed under the present experimental conditions (Figure 3C).

Figure 3 Immunoblot analysis of p36 and p46

(A, B) One representative revelation of specific bands of p36 and p46 respectively in the four experimental groups. (C) Quantification of specific bands for each group. The fed (+P_i) group was taken as control (100%). Results are mean values $\pm$ S.E.M. for each group ($n = 5-6$). Bars headed by same letters are statistically different from each other by: ^a $P < 0.02$; ^b $P < 0.02$ (unpaired Student's *t*-test).

Figure 4 Northern blot of mRNA of p36 and p46

(A, B) One representative revelation of p36 and p46 mRNA levels respectively in the four experimental groups. (C) Quantification of p36 mRNA and p46 mRNA of each group. The fed (+Pi) group was taken as control (100%). Results are mean values $\pm$ S.E.M. ($n = 3-4$). Bars headed by same letters are statistically different from each other by: ^a $P < 0.05$; ^b $P < 0.05$; ^c $P < 0.05$; ^d $P < 0.05$ (unpaired Student's *t*-test).

Northern-blot analysis of p36 and p46 G6Pase mRNA

In order to know if the increased p36 protein was due to increased synthesis or decreased degradation, the p36 mRNA abundance was measured. Northern blots of p36 mRNA and p46 mRNA were performed with the full-length cDNA of p36 or p46 as a probe respectively. Figure 4(A) shows a representative blot for p36 in the four experimental groups, and Figure 4(C) shows the quantification of the bands. It can be seen that fasting increases p36 mRNA, a result consistent with the corresponding Western blot and which has been reported previously [16]. Also in agreement with the immunoreactivity, p36 mRNA increased significantly in the ($-P_i$)-diet groups as compared with the $+P_i$ -diet groups, showing that alimentary P_i deficiency affects p36 gene expression. The liver p46 mRNA changes were parallel with those of p36. Figure 4 shows, indeed, that p46 mRNA abundance was increased with fasting, as documented previously [18], as well as with the ($-P_i$) diet in the fed and fasted groups. The fact that these changes in mRNA were not associated with changes in protein (see Figure 3) suggests either increased degradation of p46 or less translation of p46 mRNA. Interestingly, a low- P_i diet induces a post-transcriptional effect on parathyroid-hormone-gene expression that is related to the binding of parathyroid proteins to the 3'-untranslated region of parathyroid-hormone mRNA; this low- P_i diet results in decreased stability of the transcript [19].

Conclusions

Contrary to the situation with kidney, where dietary P_i deprivation markedly induces overexpression of the Na/ P_i -2 co-transporter, this diet does not affect liver microsomal P_i transport. The present results show, for the first time, that P_i deprivation *in vivo* causes microsomal liver G6Pase up-regulation. Under our experimental conditions, an increase in the p36 catalytic subunit alone is sufficient to enhance G6Pase activity. This could, in turn, favour increased hepatic glucose production and be partially causal in the association found between hypophosphataemia and glucose intolerance.

We do not know how P_i deficiency leads to increased gene expression. Studies using cultured cells, in which the extracellular parameters can be controlled better than *in vivo*, could give us that information. It will also be interesting to see whether other enzymes, besides G6Pase, involved in glucose production by the liver, are also affected by P_i deprivation. The candidate genes to be affected by P_i deficiency are glucokinase, pyruvate kinase (that are expected to be down-regulated) and phosphoenolpyruvate carboxykinase (that is expected to be up-regulated). This work is in progress.

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