Effect of the dietary supplementation with a phosphatidyl-inositol metabolite, glycerophosphorylinositol, on Na^+/K^+ ATPase activity and body weight in normal rats

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Among the functions related to the inositol-containing phospholipids in the mammalian cells there is the activation of the Na^+/K^+ ATPase. A reduction of the activity of this enzyme is implicated in the reduced motor nerve conduction velocity observed in diabetic rats, in which concentration of free inositol in the peripheral nerve is lower than in normal rats. Even erythrocytes from diabetics have fewer sodium pumps than do erythrocytes from age-matched controls.

Feeding normal, weaning rats glycerophosphorylinositol (GPI), a metabolite of myo-inositol, we investigated the connections among inositol availability, sodium pump activity, and development. Unexpectedly, GPI supplementation determined a reduction in the Na^+/K^+ ATPase activity and a decrease in the phosphatidylinositol content in the erythrocyte membranes, without altering the phosphatidylinositol fatty acid composition.

Apart from all considerations about the mechanism of action of GPI, these data clearly state the relationship between sodium pump activity and phosphatidylinositol levels.

Keywords: inositol; phosphatidylinositol; glycerophosphorylinositol; Na^+/K^+ ATPase; body weight

Introduction

The metabolism of myo-inositol has been studied extensively in mammalian cells, particularly after the recognition of a dynamic role for membrane phosphoinositides in providing for the release of the second messengers 1,2 diacylglycerol and inositoltrisphosphate in stimulated cells.

Myo-inositol is found in the mixed diets of humans in its free form, as inositol-containing phospholipids, and as phytic acid (inositol hexaphosphate).[1] It has been estimated that a mixed North American diet provides the human adult with approximately 1 g of total inositol per day.[2] In addition to dietary supplementation, a biosynthetic capacity of rat testis, brain, kidney, and liver to synthesize inositol from glucose exists.[3]

Alterations in inositol and inositol phospholipid metabolism have been implicated in various diseases although, in most cases, no definitive associations have been established.

Experimental diabetes renders rats unable to maintain normal concentrations of free inositol in the peripheral nerve, which is related to a decreased motor nerve conduction velocity. Greene and Lattimer[4] studied the phospholipid-dependent membrane-bound Na^+/K^+ ATPase in an attempt to provide a potential mechanism that would link defects in diabetic peripheral nerve to an abnormal inositol metabolism.

In mammalian cells, the membrane-bound sodium-potassium ATPase (EC 3.6.1.4.) is regulated by a variety of intrinsic intracellular modulators (including cytoplasmic sodium, potassium, magnesium, calcium, ATP, and pH), by the characteristics of the plasma membrane into which it is embedded, and by extra-

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cellular sodium and potassium concentrations.[5,6] The sodium-pump ATPase is activated by acidic phospholipids. Mandersloot et al.[7] reported that the enzyme is activated by phosphatidylinositol.

The enzyme activity has been found to be reduced in homogenates of sciatic nerve in experimental diabetes. This reduction is selectively prevented by 1% inositol supplementation, which restores normal nerve conduction.[4] Thus, the impairment of diabetic rat sodium-potassium ATPase is a consequence of altered myo-inositol metabolism, which secondarily alters the structure and/or function of the sodium-potassium ATPase-membrane complex. Because myo-inositol is the substrate for the synthesis of phosphatidylinositol (and for the higher phosphoinositides), which is an endogenous regulator for renal microsomal sodium-potassium ATPase,[7,8] it is tempting to speculate that the effect of myo-inositol on nerve sodium-potassium ATPase is mediated by this phospholipid. Support for this connection comes from the observations of Simmons et al.[9] that free myo-inositol may limit phosphatidylinositol turnover.

Dietary inositol may correct diabetic nerve conduction by exchanging enzyme activity, and this may be mediated via alterations in inositol-containing phospholipids. Many investigators suggest that oral inositol supplementation may be of benefit in the prevention and treatment of certain complications associated with human diabetes and in other diseases.[1,10]

In this study, we tried to correlate the activity of sodium-pump ATPase to the availability of inositol in normal rats. We have measured the sodium-potassium ATPase activity in erythrocyte membranes (ghosts) of normal rats, fed a standard or an experimental supplemented diet.

We chose to determine the enzyme activity on erythrocyte membranes because they are well defined, accessible, and easily purified. Furthermore, Na^+/K^+ ATPase of erythrocytes from streptozotocin diabetic rats is defective,[11] and erythrocytes from diabetics with neuropathy have fewer sodium pumps than do erythrocytes from age-matched controls.[12]

The experimental diet was supplemented with glicerolphosphorylinositol (GPI), a phosphatidylinositol metabolite which is thought to be usually formed in the intestine during phosphatidylinositol digestion and absorption.[1]

Materials and methods

Thirty-two male Wistar rats, aged 21 days, were used. The animals were divided at random into the following two groups: (1) Control group (16 animals) fed ad libitum for 30 (8 animals) or 60 (8 animals) days on a balanced and complete standard diet for rat (Dottori and Piccioni, Brescia, Italy), containing 0.2% myo-inositol; (2) Experimental group (16 animals) fed ad libitum for 30 (8 rats) or 60 (8 rats) days on the same standard diet supplemented with 100 mg/kg body weight/day GPI.

The standard diet provided 4.02 Kcal/g.

Rat body weight and the amount of food ingested by each rat were carefully recorded daily, in order to determine the amount of free inositol and GPI ingested (in mg). Food consumption ranged from 8 ± 1 g/day at the beginning and 25 ± 2 g/day at the end of the study. Therefore, at the beginning of the study, control rats received about 16 mg free-inositol daily, while treated rats received 16 mg free-inositol plus 4.5 mg GPI daily. At the end of the 60-day treatment, control rats received 50 mg free-inositol daily, while treated rats received 50 mg free-inositol plus 30 mg GPI. 1 mg GPI corresponds to 0.54 mg free-inositol.

GPI was a kind gift from Depha Team s.r.l. (Milano, Italy), and its purity was about 99%.

Animals were housed in individual cages, in strictly controlled conditions of temperature (20 ± 2° C) and humidity (60%–70%), with a dark:light cycle of 12 hr. Water was provided ad libitum.

Rat body weight was recorded every day, and some biohumoral parameters (glycemia, azotemia, GOT, GPT) were determined on venous blood at the end of the trial.

After 30 or 60 days, rats were killed by decapitation and venous blood collected.

Red cell ghosts were obtained by hypotonic hemolysis from venous blood by the method of Dodge et al.[13] Red cell membrane Na^+/K^+ ATPase. (Na^+ plus K^+-activated ATP phosphohydrolase, E.C. 3.6.1.4) activity was measured on isolated ghosts by evaluating the inorganic phosphorus according to Emmelot.[14] Briefly, the enzyme assay was carried out with an aliquot of erythrocyte membrane preparation corresponding to 0.2 mg protein incubated with 2.0 mL of 80 mmol/L NaCl, 34 mmol/L KCl, 2 mmol/L $MgCl_2$, 25 mmol/L Tris buffer pH 7.4, 1 mmol/L ATP, in the presence or in the absence of 1 mmol/L ouabain. Enzyme activity was expressed as μmol Pi released × h^{-1} × mg $protein^{-1}$.

In rats treated for 30 days, ghost phosphatidylinositol fatty acid composition was analyzed. Total lipids were extracted from ghosts according to Folch et al.[15] Phosphatidylinositol fraction was separated from total lipids by TLC using plates previously impregnated with 1% potassiumoxalate in methanol/water (2:3 vol/vol) and activated (15 min, 110° C). The plates were developed in chloroform/acetone/methanol/acetic acid/water (40:15:13:12:8 vol/vol) as described.[16] The phosphatidylinositol spot was visualized with iodine vapor and identified by comparison with cochromatographed standard. After extraction of the scraped spots, 15:0 fatty acid (10 nmol) as an internal standard was added, and the samples were methylesterified using methanol/HCl as reported by Stoffel.[17] The resulting methyl esters were gas chromatographed to obtain the phosphatidylinositol fatty acid composition, on a C.Erba mod. 4160 gas chromatograph equipped with a 10 m + 0.32 mm i.d. glass capillary column coated with SE52 stationary phase, as previously described.[18]

All reagents were of analytical grade. Data are expressed as means ± SD; statistical analysis was performed using the Student's *t* test.[19]

Results

In spite of similar food intake and similar body weight at the beginning of the trial, rats fed the experimental diet had a higher increase in body weight during the weeks 2–4 of treatment than the control rats ($P < 0.05$) (*Figure 1*). These differences disappeared during the following period of treatment (data not shown). No differences were detected in the biohumoral parameters measured between the two groups after both 30 and 60 days of treatment (data not shown).

The Na^+/K^+ ATPase activity (expressed as μmol

of inorganic phosphorus released per hour per mg erythrocyte membrane protein) in the control and the experimental groups fed for 30 and 60 days is reported in *Figure 2*. In the rats fed GPI for 30 days there was a significant decrease in the Na^+/K^+ ATPase activity in comparison with the control group ($P < 0.05$). No differences were detected after the 60 days treatment between the two groups.

PI fatty acid composition of the control and the experimental rat ghosts after 30 days of treatment is shown in *Table 1*. No significant differences were detected between the two groups in the PI fatty acid composition, while PI content (expressed as nmol of fatty acids esterified in the PI fraction/mg protein) in red cell membranes of the experimental rats was lower than in the control rats ($P < 0.05$) (*Table 2*). On the contrary, no differences in ghost PI content were detected between the two groups after 60 days of treatment (*Table 2*).

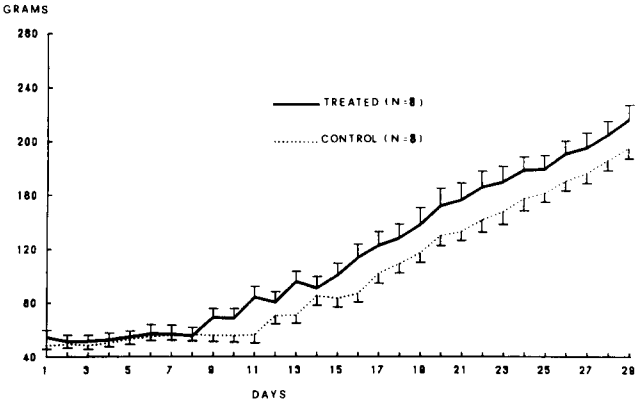

Figure 1 Body weight modifications of control and treated rats during the 30 days trial. Data are means ± SD; the number of rats examined is reported within the brackets. Statistical analysis was performed using the Student's *t*-test: since day 9 to day 30 (apart from day 12 and 14) the comparison between control and treated rat body weight was statistically significant (at least $P > 0.05$).

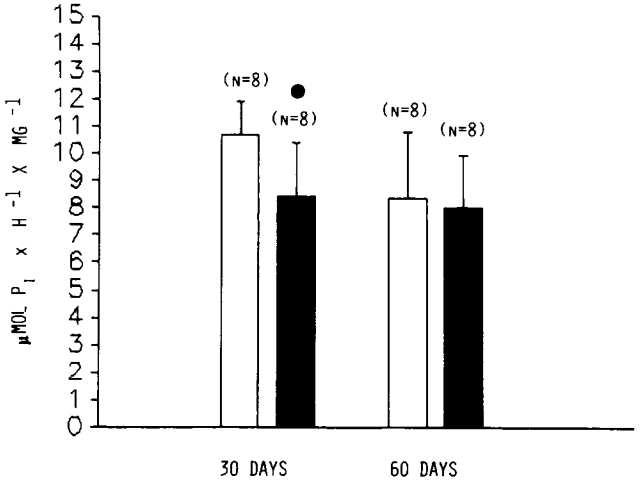

Figure 2 Na^+/K^+ ATPase activity (μmol Pi × h^{-1} mg $protein^{-1}$) of erythrocyte membrane from control (blank bars) and treated rats (filled bars) after the 30 and 60 day trials. Data are means ± SD; the number of rats examined is reported within the brackets. Statistical analysis was performed using the Student's *t*-test: 30 day trial, control vs treated = $P < 0.05$; 60 day trial, control vs treated = NS.

Table 1 PI fatty acid composition (nmol/100 nmol) of erythrocyte membranes from control and treated rats after the 30 days trial

Fatty acid	Control (n = 8)	Treated (n = 8)
12:0	1.35 ± 0.15	1.48 ± 0.32
14:0	1.44 ± 0.19	1.20 ± 0.26
16:0	32.97 ± 4.73	30.31 ± 6.27
16:1	2.08 ± 0.69	1.80 ± 0.27
17:0	0.65 ± 0.47	0.47 ± 0.34
18:0	18.67 ± 3.69	18.04 ± 2.65
18:1	9.85 ± 2.69	11.34 ± 4.36
18:2	3.40 ± 0.38	3.57 ± 0.93
20:3	0.86 ± 0.39	0.89 ± 0.37
20:4n-6	23.34 ± 4.32	26.47 ± 4.71
22:4n-6	4.20 ± 2.30	2.61 ± 0.90
22:6n-3	1.19 ± 0.55	1.82 ± 0.72

Data are means ± SD; the number of rats examined is reported within the brackets. Statistical analysis was performed using the Student's *t*-test: NS.

Table 2 PI content (expressed as nmol of fatty acids esterified in the PI fraction/mg protein) of erythrocyte membranes from control and treated rats

	30 Days trial	60 Days trial
Control	70.15 ± 6.32 (n = 8)	68.73 ± 4.12 (n = 8)
Treated	55.42 ± 11.22° (n = 8)	69.48 ± 5.16 (n = 8)

Data are means ± SD; the number of rats examined is reported within the brackets. Data were obtained by gas chromatography adding 10 nmol of an internal standard (C 15:0) to each sample examined. Statistical analysis was performed using the Student's *t*-test: 30 days, control vs treated = ° $P < 0.05$; 60 days, control vs treated = NS.

Discussion

Quantitatively, the most impressive abnormality in many tissues in diabetes is a diversion of glucose metabolism from oxidative to reductive pathways; when hyperglycemia is present, excess glucose enters the cells, glycolytic pathways become saturated, and there is a marked increase in the reduction of sorbitol by the enzyme aldose reductase. In virtually every tissue prone to diabetic complications, a rise of sorbitol level is accompanied by evidence of myo-inositol depletion within the tissue; however, the exact mechanism for this seesaw interrelationship is still not clear.[10] Furthermore, concentration-dependent activation of the sorbitol pathway by glucose leads to inactivation of Na^+/K^+ ATPase in nerve and other tissues.[20] Despite its close empirical association with myo-inositol depletion, the Na^+/K^+ ATPase defect is not well understood in mechanistic terms.

Myo-inositol may limit the availability of phosphoinositide-derived diacylglycerol (DG), and DG-mediated stimulation of protein-kinase C (PKC) may regulate Na^+/K^+ ATPase activity. Therefore, an inositol

dietary supplementation should restore the sodium pump activity by increasing the synthesis of phosphoinositides and, consequently, of the protein-kinase C activator, DG.

The aim of this study was to determine the effect of dietary supplementation with a myo-inositol metabolite, GPI, on ghost Na^+/K^+ ATPase from normal rats. We chose to supplement the diet with GPI, instead of free inositol, because we hypothesized that it could be more rapidly metabolized to PI than free-inositol, and because we were willing to test a new, not commercially available compound. We thought that GPI could determine a positive effect on the sodium pump activity, via an enhanced availability of PI and PI derivatives.

Unexpectedly, feeding weaning rats GPI for 30 days determined a decrease of Na^+/K^+ ATPase activity; this observation fits very well with the higher increase of body weight observed in these rats, when compared with control rats, not accompanied by alteration in food consumption. In fact, several authors[21,22] have shown a decrease in the sodium pump activity during the onset of obesity. The physiologic significance of the decrease is unknown, but it is reasonable to suspect that it might facilitate the maintenance of the increase of body weight considering the lower energy expenditure subsequent to the reduced activity of this enzyme.[23]

Conversely, no differences in both Na^+/K^+ ATPase activity and increase of body weight were detected after the 60-day treatment between control and treated rats. A decrease in the Na^+/K^+ ATPase activity was found in both 60 day-controls ($P < 0.05$) and 60 day-treated rats ($P < 0.02$) compared to 30 day-controls. This diminished activity could be accounted to a well-established effect of aging; in fact, many authors[24-28] report, in different cells and different animal species, an age-dependent reduction in the sodium pump activity.

Because a close correlation between sodium pump and phosphatidylinositol exists, and it is well proven that the amount and composition of the lipids affect the kinetic properties of most membrane-bound enzymes, we determined the erythrocyte PI fatty acid composition.

The 30-day GPI supplementation did not alter PI fatty acid composition in the red cell ghosts, but it determined a reduction in PI content of these membranes. Conversely, ghost PI content was similar between control and treated rats after the 60-day GPI supplementation. Therefore, we can affirm that, after 60 days of treatment, erythrocyte membrane PI content comes back to its "normal" level in the treated rats (compared to both 30- and 60-day controls), and the activity of the sodium pump does the same (compared with the activity of the 60-day-treated rats). As mentioned above, the decrease in the Na^+/K^+ ATPase activity in the 60-day rats, regardless of the dietary treatment, compared to the 30-day controls is a physiologic effect of aging, independent of PI level.

A function for PI in regulating enzyme activities and transport processes is supported by evidence of specific interactions between this phospholipid and protein; particularly, PI is recognized as an endogenous activator of the Na^+/K^+ ATPase. Therefore, it is reasonable that the observed decrease in ghost PI content could determine the detected impairment in Na^+/K^+ ATPase activity.

Many authors[29-31] failed to support the hypothesis that the late complications of diabetes are mediated through decreased total tissue myo-inositol, secondary to chronic hyperglycemia. On the other hand, Zhu and Eichberg[32] demonstrated a decreased incorporation of ^{3}[H] myo-inositol into PI in the peripheral nerve from experimental diabetic rats, and hypothesized the presence of a small pool of myo-inositol that is not in equilibrium with the bulk of cell myo-inositol and that is preferentially utilized for PI synthesis. Therefore, cells could present a decreased PI content even in the presence of a normal myo-inositol level.

Furthermore, Macaulay and Larkins[33] and Thakkar et al.[34] demonstrated the presence of a class of inositolphosphates containing oligosaccharides, derived from membrane glycanphosphatidylinositol precursors (PI-gly), which is a possible mediator of insulin action. This PI-gly represents only a minor proportion of the total cellular phospholipid, and its insulin-stimulated turnover seems to play a significant role in insulin signal transduction. Defects in synthesis or breakdown of this compound may be a part of the impaired insulin action in diabetes.

The mechanism by which feeding GPI determined a reduction in ghost PI content is unclear. Little is known about the mode of digestion and absorption of GPI; it could be reacylated to lysoPI or further hydrolyzed to myo-inositol. Greene et al.[35] reported that an excessive elevation in plasma inositol levels, induced by feeding a diet containing 3% inositol, decreases the motor nerve conduction velocity, in both normal and diabetic rats, probably via a reduction in Na^+/K^+ ATPase activity. The dose of GPI we used was quite low, but it could have determined the production of an amount of free inositol able to interfere with the sodium pump activity. This excess of myo-inositol could also have interfered with the synthesis of PI, leading to the observed decrease in ghost PI content. Alternatively, it is possible to hypothesize an inhibitory effect of GPI, even at a low level, on PI synthesis. In both the hypotheses, it is not clear the mechanism by which, after 60 days of treatment, all the differences in both sodium pump activity and erythrocyte membrane PI content disappear.

Apart from these considerations, our data clearly show evidence of a very close relationship between Na^+/K^+ ATPase activity and PI level in erythrocyte membranes.

It is important to consider that we used normal rats, in which free-inositol levels are presumed to be normal. Further studies are needed to understand the role of these related metabolic dearrangements and the importance of an inositol dietary supplementation in diabetic and/or inositol deficient animals.

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