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1-DEOXYNOJIRIMYCIN AND RELATED COMPOUNDS INHIBIT GLYCOGENOLYSIS IN THE LIVER WITHOUT AFFECTING THE CONCENTRATION OF PHOSPHORYLASE *a*

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Abstract—Administration *in vivo* of the α-glucosidase inhibitors 1-deoxynojirimycin and its derivatives BAY m 1099 (miglitol) and BAY o 1248 resulted in a dose- and time-dependent decrease in the rate of hepatic glycogenolysis induced by glucagon. This represents a direct effect on the liver, since it could be reproduced on isolated hepatocytes. The amount of glucose produced by hepatocytes over a period of 10–20 min after addition of glucagon was decreased by about 70, 60 and 45% in the presence of maximally effective concentrations of BAY o 1248, deoxynojirimycin, and BAY m 1099, respectively. Half-maximal effects were observed at inhibitor concentrations between 20 and 100 μM. The concentrations of phosphorylase *a* and glycogen synthase *a* were not affected by inclusion of the α-glucosidase inhibitors in the hepatocyte suspensions. Thus, the antiglycogenolytic action of these compounds is not mediated by an altered activation state of the rate-limiting enzymes of glycogenolysis and of glycogen synthesis.

In recent years several drugs have been developed with an antihyperglycemic action when administered *per os* [1–3]. Most of these compounds act by inhibition of intestinal α-glucosidases, thus preventing the breakdown of oligosaccharides into absorbable monosaccharides. In contrast to, for example, acarbose (a pseudo-tetrasaccharide), the glucose analogue 1-deoxynojirimycin (dNOJ) and some of its *N*-substituted derivatives (BAY m 1099 and BAY o 1248) are completely absorbed from the intestines [2]. As a consequence, these products can also exert direct effects on internal tissues. Such a mechanism has been invoked to account for the observation that orally taken BAY m 1099 enhances the disposal of blood glucose after a glucose load [4]. Other work has also shown that dNOJ inhibits markedly the secretion of glycosylated proteins by cultured hepatoma cells [5], apparently as a result of its inhibitory effect on microsomal glucosidases; this compound is therefore widely used as a tool to study the role of oligosaccharide trimming in the synthesis and secretion of glycoproteins (see [5, 6] for references).

Glycogen breakdown in the liver can occur by two different mechanisms. The flux through the classical pathway is determined by the concentration of phosphorylase *a*, which catalyzes the production of glucose-1-phosphate from the non-reducing ends of the polymer. In addition to this phosphorolytic pathway, the liver is also equipped with enzymes like α-amylase and several α-glucosidases [7, 8], which can degrade glycogen hydrolytically. Without any doubt the phosphorolytic pathway is quantitatively much more important, and represents the primary mechanism for the control of glycogenolysis by hormonal and metabolic stimuli [9]. However, a role for a hydrolytic pathway has been proposed for the glycogenolysis observed in conditions of anoxia or in the presence of inhibitors of the respiratory chain (see [9] for references). In an attempt to check the latter hypothesis we used dNOJ to block the hepatic α-glucosidases. However, quite surprisingly we observed that this agent was a potent and general inhibitor of glycogenolysis, whether elicited by KCN, glucagon or a calcium ionophore. This indicated that dNOJ inhibits the classical, phosphorolytic pathway of glycogenolysis, and was the starting point of the present work. Part of this work has been presented in a preliminary form [10].

MATERIALS AND METHODS

Chemicals. 1-Deoxynojirimycin, *N*-hydroxyethyl-1-deoxynojirimycin (miglitol, BAY m 1099), *N*-[β-(4-ethoxycarbonylphenoxy)-ethyl]-1-deoxynojirimycin (BAY o 1248) and acarbose were kindly provided by Dr. F. Seuter (Bayer AG). Glucagon and insulin (Actrapid) were commercial pharmaceutical preparations from Novo Industri. Sodium pentobarbital (Nembutal) was likewise obtained from Abbott. The calcium ionophore A23187 was purchased from Boehringer.

Handling of animals and livers. Male Wistar rats of about 250 g were used throughout this work. The *in vivo* experiments were performed on overnight sucrose-fed rats. Around 11:00 hr the rats were anaesthetized by an intraperitoneal injection of

sodium pentobarbital (12 mg). After 15 min dNOJ (25 mg in 0.5 ml 0.15 M NaCl) was injected in the dorsal penile vein, and the abdomen was opened. After an additional 5 min, an intensive hepatic glycogenolysis was induced by a subcutaneous injection of an isotonic solution (0.5 ml) of glucagon (60 μg) and an overdose of insulin (2 I.U.). At the indicated times a liver lobule was isolated by a ligature and dropped in 3 volumes of a solution of KOH (30%) for the assay of glycogen. The *in vivo* experiments with BAY m 1099, BAY o 1248 and acarbose were performed in a similar way, except for the following: the animals did not have access to food or water from 8:00 hr onwards; and the indicated doses of the inhibitors, dissolved in 2 ml of water (BAY m 1099 and acarbose) or water containing 2 μmol HCl/μmol compound (BAY o 1248), were administered by an intragastric tube, 2 hr before the injection of pentobarbital.

Isolation and incubation of hepatocytes. The cells were prepared in the morning from normally fed rats; the preparation and incubation were as previously described [11]. After a preincubation for 15 min in a Krebs–Henseleit bicarbonate buffer containing bacitracin (1 mg/ml) and 5 mM glucose, a high rate of glycogenolysis was induced by the addition of 1 μM glucagon or, occasionally, by 10 μM of the calcium ionophore A23187. The α-glucosidase inhibitors, dissolved in water or in dimethylsulphoxide (BAY o 1248), were added at the beginning of the preincubation period unless explicitly stated otherwise. At the indicated times a sample of the suspension was added to an equal volume of ice-cold isotonic NaCl and centrifuged (2 sec at 10,000 *g*) for the assay of glucose in the supernatant. In the present conditions glucose production corresponds quantitatively to glycogenolysis [9]. Samples for the assay of glycogen synthase and phosphorylase were handled as reported earlier [11]. It has been checked that the final concentration of dimethylsulphoxide (1%, v/v) in the experiments with BAY o 1248 did not affect the integrity of the cells, the rate of glucose production, and the concentrations of phosphorylase *a* and of glycogen synthase *a*.

Assays. Glucose was determined according to the method of Dahlqvist [12]. Glycogen was assayed as described previously after digestion of the biological specimens in hot KOH [9]. Phosphorylase [9] and glycogen synthase [13] were assayed as described. The protein concentration was measured according to Bradford [14] with reagent from Bio-Rad Laboratories and bovine serum albumin as standard.

Statistical analyses. Results are expressed as means ± SE for the indicated number of observations. Vertical bars in the graphs (indicated where the scale permits) represent ± SE. Statistical treatment was by Student's *t*-test for either paired or independent random samples, according to the experimental design.

RESULTS

Administration of α-glucosidase inhibitors in vivo

In these experiments the mean concentration of glycogen varied in different groups between 50 and 70 mg/g liver before the induction of glycogenolysis.

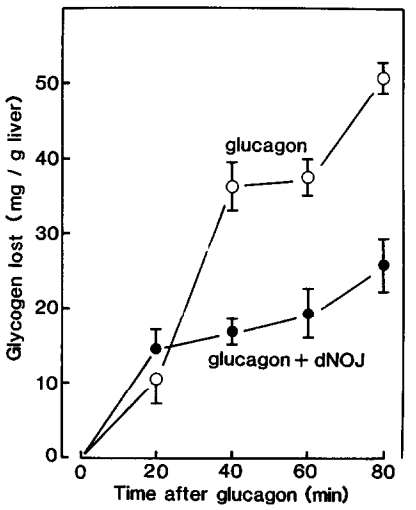

Fig. 1. Inhibition of hepatic glycogenolysis *in vivo* by 1-deoxynojirimycin. 25 mg dNOJ or solvent were injected 5 min before the administration of glucagon plus insulin (5 animals/group). At the indicated times liver samples were taken for the assay of glycogen.

The injection of glucagon plus insulin resulted in a fast and steady loss of hepatic glycogen, which amounted to about 50 mg/g liver during the ensuing 80 min (Figs 1 and 2). When dNOJ had been injected 5 min before glucagon, the rate of glycogenolysis was not affected for the first 20 min, but thereafter it was decreased by about 75% (Fig. 1; $P < 0.002$).

An impairment of the hepatic glycogenolysis was also evident when the *N*-substituted derivatives of dNOJ had been given intragastrically (Fig. 2). Within the investigated range (1.25–125 mg/rat) BAY o 1248 was a much more powerful inhibitor than was BAY m 1099; at the highest dose it caused a virtual arrest of glycogenolysis from 40 min onwards. It should be noted that the effect of these inhibitors increased clearly with time, even though they had been administered more than 2 hr before the onset of glycogenolysis. Acarbose did not significantly affect glycogenolysis, even at 125 mg/animal (not illustrated).

Effects on isolated hepatocytes

Figure 3 shows the influence of a maximally effective concentration of deoxynojirimycin on the glucagon-induced glucose production by isolated hepatocytes. The rate of glucose accumulation was constant for 50 min in the control condition, but it slowed down progressively in the presence of the inhibitor. A similar response was obtained after addition of 0.6 mM of either BAY m 1099 or BAY o 1248 (not shown). The dose-dependency of these effects is illustrated in Fig. 4. In accordance with the *in vivo* observations (*cf.* Figs. 1 and 2), BAY o 1248 was the most potent inhibitor, whereas BAY m 1099 was the weakest. Maximally effective concentrations inhibited the production of glucose by 70% (BAY o 1248), 60% (deoxynojirimycin), and 45% (BAY m 1099). In contrast, the half-maximal effect occurred

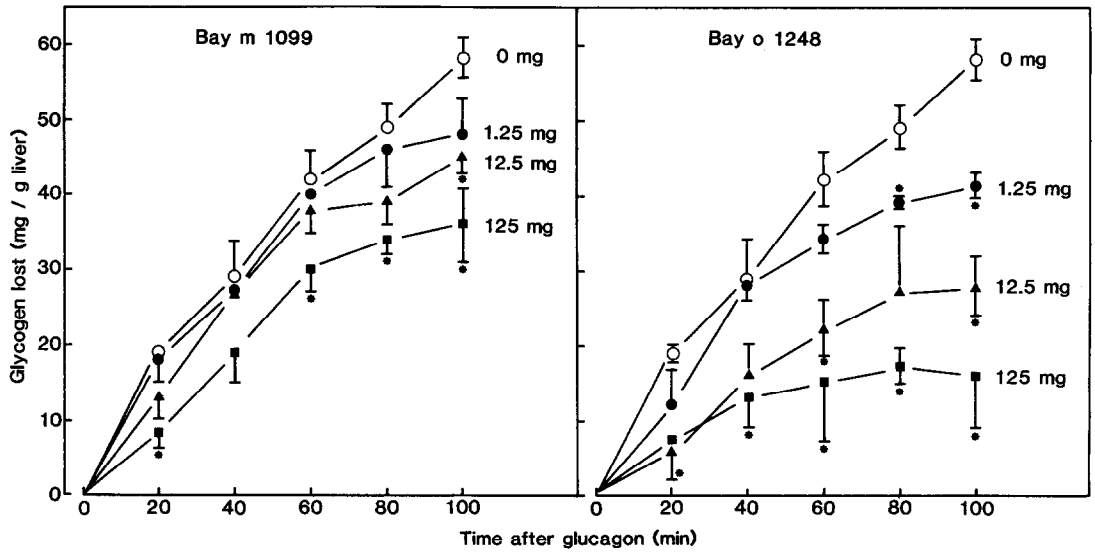

Fig. 2. Effects of intragastric administration of BAY m 1099 and BAY o 1248 on hepatic glycogenolysis *in vivo*. The test substances were administered at the indicated dose per rat, 140 min before the induction of hepatic glycogenolysis with glucagon plus insulin. At the indicated times liver samples were taken for the assay of glycogen. There were 5 animals in each group. Asterisks indicate values that differ significantly from the control group ($P < 0.05$ or smaller).

at closely similar inhibitor concentrations (between 20 and 100 μM).

Other experiments (not illustrated) showed that the inhibitory effect of dNOJ was equally important, whether glycogenolysis was induced by glucagon or by 1 mM KCN. Furthermore, in the experiments illustrated in Fig. 5, where glycogenolysis was induced by a calcium ionophore, the production of glucose was decreased by 41% in the presence of dNOJ and by 62% in the presence of BAY o 1248. In contrast, the low rate of glucose production in the absence of any glycogenolytic agent was little affected by dNOJ (not illustrated). There is a small contribution of α-amylase to the overall glycogenolysis in hepatocyte suspensions, owing to the presence of some damaged cells; this contribution can be cancelled by addition of the α-amylase inhibitor BAY e 4609 [9]. However, the latter compound (0.1 mg/ml) did not modify the effects of dNOJ.

Since there is a close structural resemblance

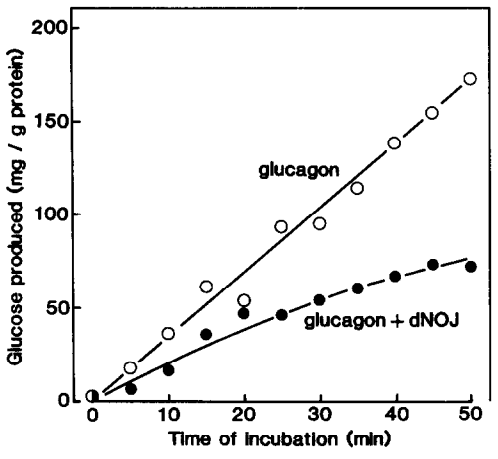

Fig. 3. Anti-glycogenolytic action of 1-deoxynojirimycin in isolated hepatocytes. At 0 min either 1 μM glucagon or glucagon plus 0.6 mM dNOJ was added to the cell suspension. At the indicated times samples were taken for the assay of glucose in the incubation medium. Results from a representative experiment.

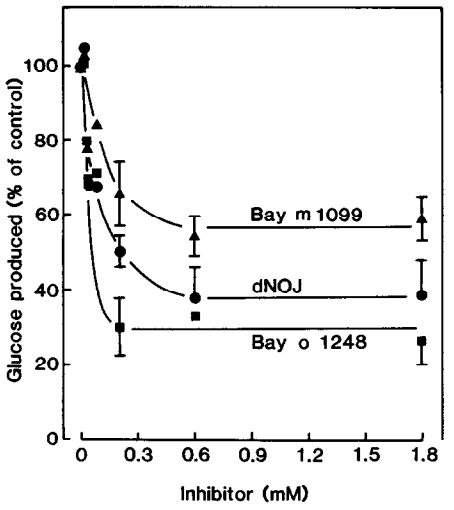

Fig. 4. Concentration-dependent inhibition of glycogenolysis in isolated hepatocytes by 1-deoxynojirimycin and its derivatives. The cells were preincubated for 15 min in the absence or presence of α-glucosidase inhibitors at the indicated concentrations. Glucagon (1 μM) was then added, and the extracellular glucose concentration was measured over the next 10 to 20 min. The amount of glucose produced in each condition is expressed as a percentage of the value recorded in the absence of inhibitor. Results from 3 hepatocyte preparations.

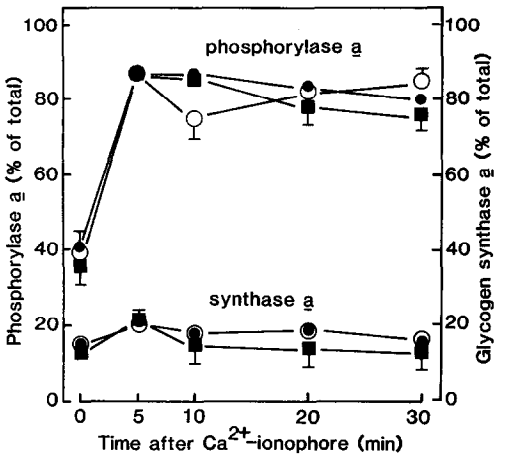

Fig. 5. Effect of the α-glucosidase inhibitors on the activities of phosphorylase and glycogen synthase in isolated hepatocytes. After a preincubation for 15 min the calcium ionophore A23187 (10 μM) was added, without (○) or with 0.6 mM of either dNOJ (●) or BAY o 1248 (■). At the indicated times samples were taken for the assays of phosphorylase and of glycogen synthase. Results from 3 hepatocyte preparations. None of the values recorded in the presence of the α-glucosidase inhibitors differed significantly from the corresponding control value ($P > 0.1$).

between dNOJ and glucose, we have also investigated whether glucose interfered in any way with the action of the inhibitor. However, the anti-glycogenolytic effect of dNOJ was equally pronounced in the absence of added glucose and in the presence of 10 mM glucose (not shown).

The activity of phosphorylase and glycogen synthase

Most known inhibitors of hepatic glycogenolysis act by decreasing the concentration of phosphorylase *a*. However, none of the α-glucosidase inhibitors had an effect on the concentration of phosphorylase *a* in isolated hepatocytes, whether glycogenolysis was induced by a calcium ionophore (Fig. 5) or by 1 μM glucagon or 10 μM dibutyryl-cAMP (not illustrated).

It might be argued that a decreased rate of glucose production could reflect unchanged glycogenolysis if simultaneous glycogen synthesis would be induced. Such a futile cycle appears to exist in the liver of the starved animal, where an important quantity of both glycogen synthase and phosphorylase are present in the active form (see [15]). However, glycogen synthase remained inactive in the presence of the α-glucosidase inhibitors when glycogenolysis was induced by the calcium ionophore (Fig. 5). The same observations were made when glucagon or dibutyryl-cAMP was used as the glycogenolytic agent (not shown).

DISCUSSION

Nojirimycin differs from β-D-glucose only in that the pyranose ring is closed by a nitrogen instead of an oxygen bridge. It can be assayed quantitatively with glucose oxidase as well as glucose dehydrogenase [3]. Glucose inhibits hepatic glycogen breakdown by binding to phosphorylase *a*. This induces a conformational change that inhibits the catalytic activity of the enzyme to some extent, but, more importantly, makes the enzyme a better substrate for inactivation by protein phosphatases (see [15]). The most efficient glucose analogue in the inactivation of phosphorylase was anhydroglucitol (1-deoxyglucose) [16], which in turn is analogous to 1-deoxynojirimycin. Yet, it is clear that the inhibition of hepatic glycogenolysis by dNOJ and its derivatives is not due to an inactivation of phosphorylase. It must also be pointed out that dNOJ was effective at less than 0.1 mM, whereas the control of phosphorylase by glucose requires concentrations in the 5–50 mM range.

The absence of an effect of the α-glucosidase inhibitors on glycogen synthase also indicates that the earlier reported effect of BAY m 1099 to accelerate the disposal of blood glucose after a glucose load [4] is not due to a facilitation of hepatic glycogen synthesis.

The present study shows also that it is a questionable approach to use α-glucosidase inhibitors to distinguish between hydrolytic and phosphorolytic mechanisms of glycogenolysis. The recent proposal that α-glucosidases play a major role in the glycogenolysis in fetal lung cells, largely because the breakdown of glycogen was inhibited by acarbose [17], may have to be reevaluated in this context.

Several mechanisms can be envisaged for the anti-glycogenolytic action of the α-glucosidase inhibitors. They could act directly or require transformation in the hepatocyte to an active compound. The target enzyme of the inhibitory substance could be any enzyme involved in the conversion of glycogen to glucose, i.e. phosphorylase *a*, debranching enzyme, phosphoglucomutase or glucose-6-phosphatase. Whatever the mechanism, it should provide an explanation for the slow development of the inhibitory effect on hepatic glycogenolysis.

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REFERENCES

1. E. Truscheit, W. Frommer, B. Junge, L. Müller, D. D. Schmidt and W. Wingender, *Angew. Chem.* **93**, 738 (1981).
2. W. Puls, H. P. Krause, L. Müller, H. Schutt, R. Sitt and G. Thomas, *Int. J. Obes.* **8**, Suppl. 1, 181 (1984).
3. L. Müller, in *Biotechnology* (Eds. H.-J. Rehm and G. Reed), Vol. 4, p.1. VCH Verlagsgesellschaft, Weinheim (1986).
4. P. H. Joubert, G. N. Foukaridis and M. L. Bopape, *Eur. J. clin. Pharmac.* **31**, 723 (1987).
5. J. B. Parent, T.-K. Yeo, K.-T. Yeo and K. Olden, *Mol. Cell. Biochem.* **72**, 21 (1986).
6. V. Gross, T.-A. Tran-Thi, R. T. Schwarz, A. D. Elbein, K. Decker and P. C. Heinrich, *Biochem. J.* **236**, 853 (1986).
7. W. J. Rutter and R. W. Brosemer, *J. biol. Chem.* **236**, 1247 (1961).
8. N. Lejeune, D. Thinès-Sempoux and H.-G. Hers, *Biochem. J.* **86**, 16 (1963).
9. A. Vandebroeck, M. Bollen, H. De Wulf and W. Stalmans. *Eur. J. Biochem.* **153**, 621 (1985).

10. M. Bollen, A. Vandebroeck and W. Stalmans, *Arch. Int. Physiol. Biochim.* **95**, B4 (1987).
11. M. Bollen, L. Hue and W. Stalmans, *Biochem. J.* **210**, 783 (1983).
12. A. Dahlqvist, *Biochem. J.* **80**, 547 (1961).
13. F. Doperé, F. Vanstapel and W. Stalmans, *Eur. J. Biochem.* **104**, 137 (1980).
14. M. M. Bradford, *Analyt. Biochem.* **72**, 248 (1976).
15. W. Stalmans, M. Bollen and L. Mvumbi, *Diabetes/Metabolism Rev.* **3**, 127 (1987).
16. W. Stalmans, M. Laloux and H.-G. Hers, *Eur. J. Biochem.* **49**, 415 (1974).
17. J. R. Bourbon, E. Doucet and M. Rieutort, *Biochim. biophys. Acta* **917**, 203 (1987).