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Daily amylin replacement reverses hepatic glycogen depletion in insulin-treated streptozotocin diabetic rats

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In streptozotocin-diabetic rats treated with insulin replacement, liver glycogen is some 35% depleted. Five consecutive daily subcutaneous injections with amylin dose-dependently restored liver glycogen to normal levels. Significant increases over insulin-only therapy occurred with amylin doses of 10, 30 and 100 μg/day, representing amylin: insulin ratios of 0.22, 0.75 and 2.79.

Amylin; Insulin; Cori cycle; Counterregulation; Streptozotocin; Type 1 diabetes

1. INTRODUCTION

Amylin [1] is a 37 amino-acid hormone co-localized and co-secreted from pancreatic β-cells along with insulin. Available data, including isolated perfused pancreas studies [2] and peripheral plasma levels [3] suggest that amylin is probably secreted in the range of 5–25% of the rate of insulin.

Amylin has marked effects on carbohydrate metabolism in vitro and in vivo. Effects on isolated skeletal muscle include inhibition of glucose incorporation into glycogen [4], inhibition of glycogen synthase [5], conversion of glycogen phosphorylase to the active *a*-form [5,6], reduction of muscle glycogen content [7], and stimulation of lactate production [4]. In vivo, amylin increases plasma lactate [8], increases endogenous glucose production [9], and increases plasma glucose [7,8].

The increases in plasma lactate and glucose in the fasted rat support the proposition that amylin regulates fuel interconversion via the Cori cycle (hepatic glycogen → plasma glucose → muscle glycogen → plasma lactate → hepatic glycogen [10]). It has been reported that this 'indirect' pathway is the predominant mechanism of hepatic glycogen synthesis in the rat [11], and it is also likely to be a major pathway in man [12].

In animal models of type 1 diabetes mellitus, in addition to insulin deficiency, there is marked amylin lack [2,13–15]; so too in the human disease [16] (Koda, J. et al., unpublished). Thus, type 1 diabetes is characterized by a combined deficiency of insulin and amylin.

Rats made diabetic with the β-cell toxin streptozotocin (STZ) have a marked depletion of liver glycogen stores [17–19] that is only partly corrected with insulin treatment [19–22]. The mechanisms for this residual deficit are unclear. We considered that it could be related to amylin deficiency, with a concomitant deficiency in hepatic glycogen repletion via the 'indirect' pathway and might be amerliorated by amylin replacement. In the experiments reported here, we confirmed previous findings of reduced liver glycogen in STZ rats treated with insulin only, and now report that liver glycogen was dose-dependently returned to normal levels in similar rats additionally treated with subcutaneous amylin once daily.

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2. MATERIALS AND METHODS

2.1. *Animals*

There were 68 male Harlan-Sprague-Dawley rats (mass 301 ± 3 g) in 8 treatment groups. Animals were housed at 22.7 ± 0.8°C in a 12:12 hour light/dark cycle (experiments being performed during the light cycle) and fed and watered ad libitum (Diet LM-485, Teklad, Madison, WI). The rats were sacrificed for harvesting of livers 4 to 5 hours into the light cycle.

2.2. *Treatment Groups*

1. STZ diabetic, insulin-only treatment (n = 11). Animals were injected with streptozotocin (Sigma Chemical Company, St Louis, MO: Sigma S0130) dissolved in water in a dose of 65 mg/kg into the lateral tail vein. Upon exhibiting 5% glycosuria (Chemstrip uGK, Boehringer-Mannheim, Germany), rats were commenced upon a sliding-scale daily insulin treatment regime (Humulin-Ultralente, Eli Lilly, Indianapolis, IN) aimed towards maintaining aketonuria (by Chemstrip) but 5% glycosuria. Maintaining diabetic rats in this metabolic state optimizes survival. Following one week of established diabetes, animals received once daily s.c. injections of amylin vehicle (water for injection) for 5 days given at the time of the insulin injection.

2.6. STZ diabetes, insulin + amylin treatment groups. These animals were treated identically to those in group 1 except that the daily subcutaneous injection contained 3 μg ($n = 5$); 10 μg ($n = 12$); 30 μg ($n = 5$); 100 μg ($n = 5$) or 300 μg ($n = 5$) of rat amylin (Bachem lot WG485[1]). The bioactivity of the peptide used in these experiments

[1]This batch of rat amylin has subsequently been found to contain a mercury contaminant, believed to be interposed between the sulphur atoms of the 2-cys 7-cys disulphide bridge (personal communication, Leighton and Cobb, Glaxo Inc, Research Triangle Park, NC, USA)

was first verified by bioassay, using inhibition of insulin-stimulated radioglucose incorporation into glycogen in the isolated stripped rat soleus muscle [4]. The EC_{50} derived for the peptide used was 6.2 nM (± 0.2 log unit). The insulin dose in each of the groups of diabetic animals averaged 1.67 U/animal/day. There were no other observable differences in the required management of the different groups.

7. Normal animals (n = 10). These animals were derived from the same stock and housed under the same conditions for the same time as those in groups 1–6, but were given no injections. 24 hours after the fifth daily injection of amylin or water (or an equivalent time after admission to the vivarium in the case of group 7 rats), the non-fasted rats were killed by decapitation and the livers immediately removed and frozen in liquid N_2, weighed and stored for subsequent determination of glycogen concentration.

8. STZ-diabetic animals, no treatment (n = 10). These animals were made diabetic as were groups 1–6, but received neither insulin nor amylin.

2.3. *Glycogen determination*

Whole livers were powdered while frozen. Approximately 200 mg of powder was further homogenized in 1.0 ml of 0.6 M perchloric acid to denature enzymes. 200 μl of the homogenate was neutralized with 0.5 vols of 1.0 M $KCHO_3$ in either of two acetate buffer solutions, one containing 2.0 ml of 200 mM acetate (pH 4.8), the other containing the same but with 18.5 U/ml of amyloglucosidase (EC5 3.2.1.3, from *Aspergillus niger*, Sigma A3423, Sigma Chemical Co., St Louis, MO) added. Following at least 20 min incubation at 23°C, the supernatants were assayed for glucose in an analyzer using D-glucose oxidase immobilized enzyme chemistry (Analyzer model 2300-STAT, YSI, Yellow Springs, OH). Purified rabbit liver glycogen (Sigma G8876) used as a standard indicated 98 ± 4% recovery of dissolved glycogen and linearity within the range of observed liver glycogen concentrations ($r = 0.9994$). All reagents were of analytical grade or better.

2.4. *Numerical methods*

Statistical comparisons were by Student's *t*-test routines contained in the SYSTAT system (Systat, Evanston, IL). All results are reported as means ± SEM.

3. RESULTS

3.1. *Liver glycogen*

Liver glycogen content was measured in rats with free access to food up to the time of sacrifice (fasting rapidly depletes liver glycogen, such that the levels after 18-h starvation are typically only 2–5% of those observed in the fed state [23]). Glycogen contents for the 8 groups of animals are shown in Fig. 1. STZ diabetic animals on no therapy showed a 67% decrease in liver glycogen concentration compared to normal rats (2.86 vs 8.6 mg/g, $P < 0.001$). STZ diabetic animals receiving insulin replacement had a 35% decrease in liver glycogen compared to normal rats (5.6 vs 8.6 mg/g, $P < 0.01$). In insulin-treated STZ diabetic rats supplemented with daily amylin, there was a dose-dependent increase in liver glycogen concentration above that in rats replaced with insulin alone ($P < 0.05$, 10 μg/day; $P < 0.02$, 30 μg/day; $P < 0.01$, 100 μg/day). The liver glycogen concentration in animals receiving 10, 30 and 100 μg amylin per day was not significantly different from that in normal animals ($P > 0.3$), although the mean values showed a dose-dependent increase with an apparent peak at 30 μg/day. The lowest dose examined, 3 μg/day did not measurably increase glycogen levels above those treated with insulin alone. Interestingly, the highest dose of amylin tested, 300 μg/day, also did not measurably restore liver glycogen towards normal, giving an apparently biphasic dose-response.

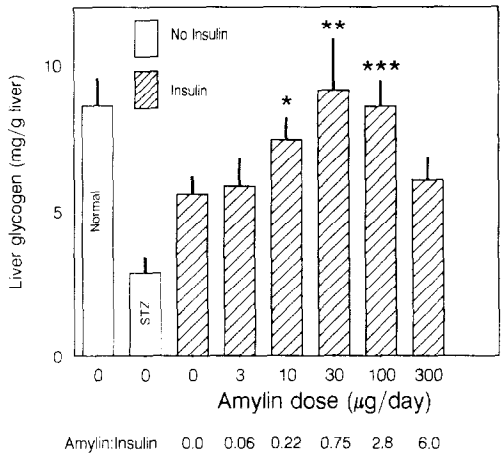

Fig. 1. Liver glycogen levels. Liver glycogen concentrations (mg/g liver) in normal rats, untreated STZ diabetic rats, STZ diabetic rats treated with insulin alone, and STZ diabetic rats treated with insulin and amylin at the daily dose indicated. Also indicated is the molar ratio of amylin to insulin. the bars represent means ± SEM. The asterisks indicate amylin-treated animals that were significantly different to those treated with insulin alone: $^{*}P < 0.05$, $^{**}P < 0.02$, $^{***}P < 0.01$. The untreated STZ diabetic group had less liver glycogen than all other groups, $P < 0.01$.

4. DISCUSSION

The results show that combined replacement of amylin and insulin can restore normal levels of liver glycogen in STZ-diabetic rats; full restoration is not achieved with insulin alone. We believe these observations to be the first demonstration of a chronic effect of amylin administration. We note that this physiological response is caused by a once-daily subcutaneous injection in a molar ratio of amylin to insulin close to that thought to occur naturally in healthy animals.

The mechanisms by which amylin restores liver glycogen in STZ-diabetic rats are not determined in the present study. However, the observations of the pattern of changes in plasma lactate and glucose following amylin administration [8], and of its glycogenolytic action [5–7], caused us to propose that amylin complements insulin in controlling Cori cycle activity [24]. Lactate is a highly interconvertable form of metabolic carbon; in the rat, it is a preferred substrate for hepatic lipogenesis [25] and is the predominant substrate for liver glycogenesis [26]. The Cori cycle may constitute an important mechanism for the interconversion and redistribution of different metabolic fuels, and amylin may drive one or more parts of this mechanism. In 1931 such a role was proposed for the catecholamines in transferring muscle glycogen to liver [27], and a similar

role was recently proposed for the redistribution of glycogen between muscles following exercise-induced depletion [28]. Amylin may share many of the actions of catecholamines except that it is present during rest.

It is notable that, as in the present study, hepatic glycogen is also normalized in islet-transplanted STZ diabetic rats [29], but not with insulin alone [19–22], suggesting the action of an islet factor other than insulin. Moreover, renal subcapsular islet transplants draining into the peripheral circulation were equally effective as splenic subcapsular transplants draining into the portal circulation; such findings are consistent with the concept that the transplanted islets provide a factor additional to insulin, which could well be amylin. Since the immediate effects of counterregulatory hormones, mainly glucagon and catecholamines, are mediated largely through hepatic glycogenolysis [30], their effectiveness is dependent upon the adequacy of liver glycogen stores. These stores, in the STZ diabetic rat at least, can apparently not be fully maintained by insulin alone. The replacement of amylin as well as insulin at appropriate doses in type 1 diabetes may be expected to promote the ability of the organism to defend itself against fuel exhaustion [31].

The reduced liver glycogen seen in the present study with the highest subcutaneous dose of amylin has been noted in normal rats treated with 0.75 or 3.0 mg subcutaneous amylin once daily (personal communication, L.A. Campfield, Hoffmann LaRoche, Nutley, NJ); this effect could be a consequence of activation of hepatic glycogen phosphorylase.

In summary, insulin-treated and untreated STZ-diabetic rats had depleted stores of liver glycogen compared to non-diabetic controls. When insulin replacement was supplemented with amylin replacement for 5 days, liver glycogen stores increased and were not different from those in non-diabetic controls. These findings support the idea that advantages may be expected from amylin/insulin co-replacement in type 1 diabetes, a disease characterized by a combined deficiency of amylin and insulin.

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