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EFFECTS OF GLP-1 AND 2,5-ANHYDRO-D-MANNITOL ON INSULIN SECRETION AND PLASMA GLUCOSE IN MICE

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ABSTRACT

The truncated glucagon-like peptide-1 (GLP-$1_{(7-36)}$amide or GLP-1) stimulates insulin secretion, enhances glucose elimination and is of potential interest in diabetes treatment. We studied the hypoglycemic action of GLP-1 in normal mice when given alone or together with the fructose analogue, 2,5-anhydro-D-mannitol (2,5-AM), which inhibits glycogenolysis and gluconeogenesis. GLP-1 (32 nmol/kg iv) lowered plasma glucose levels after 25 min to 4.6±0.2 mmol/l compared with 7.3±0.4 mmol/l in controls ($P<0.001$). Also 2,5-AM (0.5 μmol/kg iv) reduced plasma glucose levels, to 5.6±0.3 mmol/l ($P<0.01$). When given together, the glucose lowering action of GLP-1 and 2,5-AM was additive, since the 25 min glucose level was 2.8±0.2 mmol/l. At 5 min after injection, GLP-1 had increased plasma insulin levels to 693±68 pmol/l compared with 342±42 pmol/l in controls ($P<0.01$). 2,5-AM abolished this increase. Furthermore, GLP-1 (32 nmol/kg) did not affect the glycogen content, neither in the liver nor in the gastrocnemic muscle in samples taken at 30 min after injection. Moreover, in isolated islets incubated at 3.3 and 8.3 mmol/l glucose, 2,5-AM at 75 mmol/l inhibited glucose-stimulated insulin secretion ($P<0.05$) showing that 2,5-AM inhibits insulin secretion both *in vivo* and *in vitro*. We conclude that GLP-1 may reduce plasma glucose levels also to levels below the basal levels under normal conditions, and that an insulin- and liver-independent action of the peptide contributes to its hypoglycemic action in normal animals.

INTRODUCTION

During recent years, the truncated form of glucagon-like peptide-1, GLP-$_{1(7-36)}$amide, or, as it is called here, GLP-1, has been postulated to be a potential new treatment modality for diabetes (1). This suggestion is based on studies showing that GLP-1 stimulates insulin secretion and lowers blood glucose concentrations both under normal conditions and in diabetes (1-6). Furthermore, the insulinotropic action of GLP-1 is glucose-dependent (7,8) which makes hypoglycemia unlikely to evolve following administration of the peptide. This conclusion is supported by our previous study which demonstrated that in normal mice, an intravenous injection of a high dose of GLP-1 stimulates a short-lived and transient insulin secretion with no hypoglycemia during the first 10 min after injection (3).

However, we have previously also demonstrated that GLP-1 enhances the glucose elimination rate during an intravenous glucose tolerance test both in normal and in insulin-deficient alloxan-diabetic mice (9), and, similarly, that the peptide increases the insulin sensitivity during a hyperglycemic clamp in patients with insulin-deficient type 1 diabetes (1). This suggests that GLP-1 also might lower the blood glucose concentrations by a peripheral, insulin-independent, action. This could indicate that GLP-1 would reduce the glycemia also to levels below those seen basally and therefore indeed induce hypoglycemia but that this would evolve at a later time point than that seen for its insulinotropic action. In this study, we have therefore examined the time course of plasma glucose levels after an intravenous injection of GLP-1 in normoglycemic mice. We have also studied whether the fructose analogue, 2,5,anhydro-D-mannitol (2,5-AM), affects the action of GLP-1

on plasma glucose levels, since 2,5-AM has previously been demonstrated to induce hypoglycemia in mice through inhibiting gluconeogenesis and glycogenolysis and thereby hepatic glucose delivery (10). Thus, administration of 2,5-AM would, first, preclude any hepatic actions of GLP-1 and, second, the simultaneous use of 2,5-AM and GLP-1 would allow conclusions on the issue whether GLP-1 could induce severe hypoglycemia. To study the possibility that a glycogenic action of GLP-1 contributes to its hypoglycemic effect, liver and muscle glycogen content were also examined. Furthermore, during the course of this study, we demonstrated that insulin secretion in mice was inhibited by 2,5-AM. Therefore, we also studied whether such an action is mediated by a direct islet action of the substance by examining insulin secretion from isolated mouse islets.

MATERIALS AND METHODS

Animals.

Adult female NMRI mice (Bomholtgård Breeding and Research Centre Ltd, Ry, Denmark), weighing 22-25 g, were used. The animals had been kept on a standard pellet diet and tap water *ad libitum* before the experiments. The study was approved by the Ethic Committee of the Lund University.

In vivo experiments.

The mice were anesthetized with thipenthal 1.8 mg/animal injected ip. At 15 min after the ip injection, synthetic human GLP-1 (Peninsula Laboratories, St Helens, Merseyside, England; dissolved in saline with the addition of 1% gelatine)

and 2,5-anhydro-D-mannitol (2,5-AM; Sigma Chemical Co, St Louis, Mo, USA; dissolved in saline) were injected intravenously into a tail vein either alone or together. Controls were injected with saline. Blood was sampled from the retrobulbar plexus immediately before and at 5, 10, 15, 25 and 35 min after the iv injection, and plasma was immediately separated and stored at -20°C until analyses. The dose of GLP-1 which was used (32 nmol/kg) has previously been shown to maximally stimulate insulin secretion in mice (3), and the doses used for 2,5-AM (0.5 μmol/kg = 75 mg/kg) has previously been shown to induce hypoglycemia in mice (10). To study whether GLP-1 affects the muscle or liver glycogen content, specimens from liver and the gastrocnemic muscle were rapidly taken from mice killed by cervical dislocation at 30 min after an intravenous injection of GLP-1 (32 nmol/kg) or saline. The tissue was rapidly frozen and stored at -20°C.

In vitro experiments.

Islets were isolated by the collagenase digestion technique (11,12). In brief, pancreas was retrogradely filled with 3 ml of Hank's Balanced Salt Solution (Sigma Chemical Co., St. Louis, U.S.A.), supplemented with 0.3 mg/ml of Collagenase P (Boehringer Mannheim Gmbh, Germany). The pancreas was subsequently removed and incubated for 20 min at 37°C. After rinsing, the islets were handpicked under a stereomicroscope and incubated overnight in RPMI 1640 medium. The islets were then washed three times and preincubated for 30 min at 37°C in a modified Krebs-Ringer bicarbonate medium, consisting of (in mmol/l): 114 NaCl, 4.4 KCl, 1.28 $CaCl_2$, 1.5 $KHPO_4$, 0.8 $MgSO_4$, and 24 $NaHCO_3$, 10 Hepes and 3.3

glucose in 95% humified air/5% CO_2 athmosphere. The islets were then transferred to new chambers, and single islets were incubated individually in 0.1 ml of the Krebs-Ringer bicarbonate medium for 60 min at 37°C in an athmosphere of 95% air/5% CO_2 in the presence of 3.3 or 8.3 mmol/l glucose and/or 1.5, 7.5, 15 or 75 mmol/l 2,5-AM according to the respective protocols. After the 60 min incubation, aliquots of the medium (2x25 µl) were removed for insulin immunoassay.

Analyses.

The concentration of insulin in plasma and medium was determined by radioimmunoassay using guinea pig anti porcine insulin (Linco Research, St Louis, Mo, USA), ^{125}I-labelled rat insulin (Novo Nordic, Bagsvaerd, Denmark) and, as standard, rat insulin (Linco). The antigen-antibody complex was dissociated by the double antibody technique (13). Plasma glucose levels were determined with the glucose oxidase technique. Tissue glycogen content was performed in homogenized tissue as analysis of glucose after glycogen breakdown (14).

Statistics.

Values are expressed as mean ± SEM. The statistical comparisons were performed using two-way analysis of variance with the Neumann-Keul *post hoc* test. A probability level of random difference of $P<0.05$ was considered significant.

RESULTS

Studies in vivo.

The intravenous injection of GLP-1 at 32 nmol/kg was followed by a reduction in plasma glucose levels that was most marked at 25 min after the injection (Fig.1).

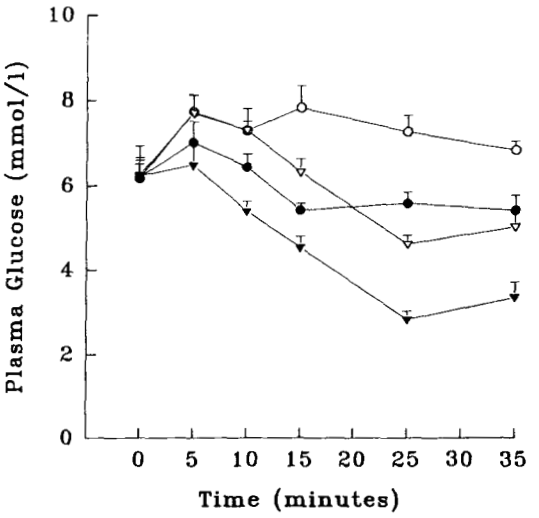

FIGURE 1

Plasma glucose levels before and after the intravenous injection of GLP-1 (32 nmol/kg; ●—●), 2,5-AM (0.5 μmol/kg;▽—▽), GLP-1 in combination with 2,5-AM (▼—▼), or in controls injected with saline (○—○). Means±SEM are shown. There were 12 animals in each group.

Similarly, 2,5-AM at 0.5 μmol/kg reduced plasma glucose levels (Fig.1). When combined, GLP-1 and 2,5-AM induced additive hypoglycemic responses. Plasma insulin levels were increased by GLP-1 at 5 min after injection (Table I) whereas at 25 min after injection, plasma insulin levels after GLP-1 were not different from that in the controls. 2,5-AM abolished the GLP-1-induced increase in plasma insulin levels. Furthermore, at 30 min after intravenous injection, GLP-1 had not affected the glycogen content neither in the liver (31.1±1.6 mg/g tissue in controls; n=12, versus 29.8±1.8 mg/g tissue after GLP-1; n=10) nor in the gastrocnemic muscle (2.6±0.4 mg/g tissue in controls; n=5, versus 1.9±0.6 mg/g tissue after GLP-1; n=5).

Table 1

Plasma insulin levels at 5 and 25 min after the intravenous injection of saline, GLP-1, 2,5-M or GLP-1 together with 2,5-AM. Means±SEM are shown. There were 12 animals in each group. Asterisks indicate the probability level of random difference versus the control group. **P<0.01.

	5 min	25 min
Saline	342±42	312±36
GLP-1 (32 nmol/kg)	693±68**	318±40
2,5-AM (0.5 μmol/kg)	380±40	390±62
GLP-1 + 2,5-AM	401±43	296±31

Studies in vitro.

When isolated mouse islets were incubated for 60 min, 2,5-AM at 75 mmol/l inhibited glucose-(8.3 mmol/l)-stimulated insulin secretion. Thus, at 8.3 mmol/l glucose, the medium insulin secretion was 7 568±567 pmol/l (n=24 batch incubations performed at 3 occassions) and this figure was reduced to 5 288±861 pmol/l by 2,5-AM at 75 mmol/l (P<0.,05; n=24). In contrast, at lower doses, 2,5-AM did not significantly affect glucose-stimulated insulin secretion.

DISCUSSION

In liver cells, the β-D-fructose analogue, 2,5-AM, is phosphorylated by fructokinase to 2,5-AM-1-phosphate which is subsequently phosphorylated by phosphofructokinase to 2,5-AM-2,6-biphosphate (15). This compound is not further metabolized but is instead accumulated inside the cells in which it inhibits both gluconeogenesis and glycogenolysis (10,15-17). Furthermore, due to its rapid phosphorylation when given *in vivo* (18), depletion of hepatic inorganic phosphate

evolves that reduces the cellular content of ATP (19). Previously, these actions of 2,5-AM have been shown to result in hypoglycemia when administered both in normal and diabetic mice and rats (10,20). We confirm in this study that 2,5-AM reduces plasma glucose levels in non-fasted normal mice.

In this study, we also demonstrate that 2,5-AM inhibits insulin secretion, both under *in vivo* conditions following induction of insulin secretion by GLP-1 and under *in vitro* conditions since glucose-stimulated insulin secretion from isolated islets was also inhibited by the drug. The inhibitory action of 2,5-AM on insulin secretion *in vivo* is most likely exerted through a direct B cell action and not indirectly through a hypoglycemic action, since it was seen already at 5 min after its administration, whereas the glucose lowering action was seen first after 25 min. On the other hand, the dose level of 2,5-AM required to inhibit insulin secretion *in vitro* was higher than *in vivo*, which could indicate partial involvement of indirect mechanisms. The mechanisms underlying the inhibition by 2,5-AM of insulin secretion remain to be studied. It may be suggested that the inhibition is caused by depletion of B cell ATP, in analogy with the action of 2,5-AM in hepatocytes (19), provided that 2,5-AM undergoes a similar metabolism in the islet B cells as in hepatocytes.

GLP-1 has previously been shown to stimulate insulin secretion and to enhance the glucose elimination in nomal mice (3,8,9). In this study, we show that GLP-1, besides increasing plasma insulin levels, also lowers the plasma glucose to levels below those seen normally in normal mice, an effect that is seen at 15 min after its intravenous administration. Furthermore, when combined with 2,5-AM, GLP-1 still lowered the glucose levels resulting in an additive hypoglycemic response of these two substances. Since 2,5-AM abolished the GLP-1-induced insulin secretion, it may be suggested that the glucose-lowering action of GLP-1 under these conditions in normal mice is exerted mainly through a peripheral, insulin-independent

action. Furthermore, since at the same time, 2,5-AM inhibits hepatic gluconeogenesis and glycogenolysis (15-17), it seems reasonable to assume that the glucose lowering action of GLP-1 is not only insulin-independent but also independent of gluconeogenesis and glycogenolysis in the liver. Such a mechanism is corroborated by direct studies on possible actions of GLP-1 on liver function, demonstrating in this study that the peptide does not affect the liver glycogen content and in other studies showing no effects by the peptide on the production of cyclic AMP (21) or glycogenolysis (22). In contrast, a stimulatory action of GLP-1 on liver glycogenesis has recently been demonstrated in isolated rat hepatocytes, which was suggested to underlie the glucose lowering action of the peptide (23). On the other hand, a study in humans has shown that GLP-1 increases the insulin-independent peripheral glucose uptake (24), which suggests a peripheral, but insulin-independent, site of action. Our present study in normal mice therefore supports this suggestion. The exact mechanism underlying the hypoglycemic action of GLP-1 still remains intriguing, however, although stimulation of peripheral glucose uptake, for example in muscles, is at present the most likely explanation. In this context, it is of interest that a recent study has demonstrated that GLP-1 binds to skeletal muscles (25).

In conclusion, this study has shown 1)that the fructose analogue, 2,5-anhydro-D-mannitol inhibits insulin secretion both *in vivo* and *in vitro*, 2)that GLP-1 may reduce plasma glucose levels also to levels below the basal levels, and 3)that the glucose lowering action of GLP-1 seems to be mediated by an insulin-independent and probably also a liver-independent action.

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