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Stabilization of insulin by alkylmaltosides. B. Oral absorption in vivo in rats

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Abstract

Enteral absorption of insulin is hampered by instability and self-association, degradation of insulin by digestive enzymes and by low macromolecular permeability. Reduction of the influence of these factors through protein stabilization should hypothetically result in increased absorption due to a higher concentration gradient of intact insulin across the intestinal mucosal barrier. Insulin in a stabilized form was shown to be absorbed after duodenal administration in normoglycemic and in diabetic rats. A homologous series of alkylmaltosides were found to stabilize insulin in solution (Hovgaard et al., 1996). For dodecylmaltoside, only minimal aggregation was observed over extended periods (60 days) under agitating conditions. In comparison, regular insulin aggregated and lost complete biological activity after 8 days. In an intraduodenal rat model, blood glucose levels were depressed to 70% of initial values and serum insulin concentrations reached 250 μU/ml. The bioavailability of stabilized dodecylmaltoside insulin was found to be 0.5–1% based on area under the curve (AUC) determination for plasma insulin levels and decreased AUC (dAUC) for blood glucose level depression.

Keywords: Insulin; Alkylmaltoside; Oral; Peptide; Drug delivery

1. Introduction

For insulin or other peptides, in general, an effective oral dosage form would be a great advantage in the treatment of many diseases. The parenteral administration of insulin is inconvenient and fails to normalize blood glucose concentrations in a number of diabetic individuals. The natural physiological release of insulin from the pancreas into the portal vein leads directly to the liver where ~50% is metabolized (Rubenstein et al., 1972). After passage through the liver, the majority of the remaining insulin is metabolized in the kidneys and in peripheral tissues (Duckworth, 1988). Most orally administered hydrophilic drugs are absorbed into the mesenteric vein which empties into the hepatic portal vein. This has also been shown to be valid for insulin (Saffran et al., 1991). The parenteral administration of insulin delivers an excess of insulin to the

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systemic circulation and fails, therefore, to mimic the natural release pathway. This may be one reason to the poor control of diabetes patients and their long-term complications. Oral insulin delivery would be pharmacologically useful if the harsh conditions of the GI tract could be overcome. Peptides are denatured in the acidic environment of the stomach and cleaved by proteolytic enzymes, such as pepsins I–III (Abe and Shigeta, 1975). In the small intestine, the peptides are subjected to attack by pancreatic enzymes, i.e. trypsin, chymotrypsin and carboxypeptidases (Silk et al., 1985). Moreover, the physical barrier that the intestinal wall represents to peptides and macromolecules absorption is well known (Mazer, 1988) and must be overcome.

Many attempts to stabilize and maintain the integrity and physiological activity of proteins and peptides have been reported. However, most attempts have produced stabilization against thermal denaturation and aggregation, particularly for insulin pump systems. Polymeric surfactants were studied by Thurow and Geisen (1984) and Chawla et al. (1985) used polyol-surfactants. The stabilization of insulin by these compounds was believed to be of a steric nature. Among other systems used are saccharides (Arakawa and Timasheff, 1982), osmolytes, such as amino acids (Arakawa and Timasheff, 1985), and water structure breakers, such as urea (Sato et al., 1983). These compounds exert their action by increasing the intramolecular hydrophobic interaction of the protein.

The absorption of intact insulin molecules from the GI-tract has been achieved by the use of potent protease inhibitors (Fujii et al., 1985; Bendayan et al., 1994) and liposomal entrapment (Rowland and Woodley, 1981). Weingarten et al. (1985) showed that association with liposomes protected insulin from enzymatic degradation. Sato (1984) showed that glycosylation of primary amino groups on insulin reduced aggregation. This was later shown to increase stability of insulin against enzymatic degradation and to increase the absorption of insulin from the small intestine (Haga et al., 1991). Similar effects were found for phenylalanine insulin derivatives by Fukushima et al. (1985) and for sulphated derivatives by Pongor et al. (1983). Mono- and dipalmitidyl insulin derivatives were absorbed from the large intestine due to increased lipophilicity (Muranishi et al., 1992).

Surfactants, fatty acids and fatty acid derivatives have frequently been used in absorption promotion of insulin from the GI tract. Sodium dodecyl sulfate (SDS) (Teng, 1986), bile salts (Kidron et al., 1982), acylcarnitines (LeCluyse et al., 1991) and fatty acids (Morimoto et al., 1983) where shown to exert their action by altering the mucosal membrane permeability. Salicylates and cyclodextrins were also shown to produce intestinal absorption (Nishihata et al., 1981; Watanabe et al., 1992). However, damage to the membrane by these treatments was a major concern (Ennis et al., 1990).

The purpose was to study the effect of alkylmaltosides on insulin stabilization and in vivo insulin absorption across the GI-tract. The alkylmaltosides were selected from the standpoint of good protein solubilization (Wheatley et al., 1984), nontoxic nature (Code of Federal Register 21, 1986), uncharged nature and hydrophobic/hydrophilic balance. Based on results from previous work by Hovgaard et al. (1996), dodecylmaltoside was chosen as the lead compound for these in vivo studies.

2. Materials and methods

2.1. Materials

Bovine insulin ($<0.5\%$ w/w Zn), gentamycin, α-acetobromomaltose (90–95% purity), octanol, decanol, dodecanol, tetradecanol, hexadecanol and octadecanol were all obtained from Sigma Chemical Co. (St. Louis, MO). Silver carbonate, iodine and silica gel 240–400 mesh, 60 Å were obtained from Aldrich Chemical Co. (Milwaukee, WI). Toluene, dichloromethane and ethylacetate were obtained from Merck (Damstadt, Germany). All chemicals were used as received without further purification.

2.2. Synthesis of alkylmaltosides

The synthesis was performed according to Hovgaard (1991). Briefly, alcohols and α-acetobromomaltose were coupled in the presence of silver carbonate. The peracetylated intermediate was isolated and deacetylated with sodium methoxide. The crude alkylmaltoside was purified by ether precipitations and preparative flash chromatography (Still et al., 1978). The products octylmaltoside (OM), decylmaltoside (DM), dodecylmaltoside (DDM), tetradecylmaltoside (TDM), hexadecylmaltoside (HDM) and octadecylmaltoside (ODM) were isolated in adequate purity for experiments. The purity determined from elemental analysis was $> 99\%$ for all compounds except ODM which was over $> 96\%$.

2.3. Blood glucose measurements

Male Sprague-Dawley rats (175–275 g, Sasco, Omaha, Nebraska) were used for oral absorption studies. On the day before an absorption experiment, a small catheter was implanted into the duodenum of the rats according to Hovgaard (1991). The rats were then fasted overnight (12–16 h) and allowed water ad libitum. To minimize the diurnal variance of the blood glucose concentrations in the animals, all experiments were performed in the morning. The rats were anesthetized with pentobarbital sodium (35 mg/ml/kg intraperitoneally) and the duodenal catheter was externalized. Blood samples were taken by jugular vein puncture using a 25 gauge needle. At specified times, 50 μl blood was collected and the glucose concentration measured using an Accu-Check IIm blood glucose monitor (Boehringer Mannheim, Indianapolis, Indiana). Two blood samples were taken prior to administration at $t = -15$ min and $t = -5$ min and averaged for the initial blood glucose value. Insulin solutions were given intraduodenally (ID) through the intestinal catheter. Doses were varied from 25 U/ml/kg to 150 U/ml/kg, stabilized with DDM in PBS of pH 7.4 in the w/w ratios 1:64 to 1:11 (insulin to DDM). Blood was sampled at 15, 30, 60, 90 and 120 min and every hour thereafter up to 6 h. As an intravenous standard, 0.5 U/ml/kg insulin was injected into the tail vein. To calculate the biological effect, decreased areas under the '% decreased blood glucose vs time' curve dAUC were calculated using the trapezoidal method. All experiments were done in at least quadruplicates.

2.4. Serum insulin measurements

Diabetes was induced in rats by injection of streptozotocin (60 mg/ml/kg) into the tail vein 7 days prior to the absorption studies. In addition, a jugular vein catheter was implanted on the day before an experiment to facilitate the collection of blood samples. The rats were fasted 12–16 h and allowed water ad libitum. To obtain sufficient serum for insulin determination, 0.4– 0.5 ml blood was collected for each sample. Saline was used for volume replacement. Blood sampling was done at 2, 4, 6, 8, 10, 12, 15, 20 and 30 min following insulin administration. Each sample was measured in duplicates using a radioimmunoassay kit for insulin (Autopak Insulin Radioimmunoassay, ICN Micromedic, Horsham, PA). Insulin was administered orally as 50 U/ml/kg and 150 U/ml/kg with DDM in the w/w ratios 1:32 and 1:11 and intravenously 0.25 U/ml/kg insulin in PBS. All experiments were performed in triplicate or quadruplicate.

3. Results and discussion

3.1. Blood glucose levels

The initial blood glucose levels of the rats varied between 3.56 mmole/l to 6.28 mmole/l. Data for each animal was normalized to its own initial blood glucose concentration values. Control experiments for oral absorption in rats are shown in Fig. 1. The blood glucose concentration decreased in the test experiments. The graph insert shows dAUC for blood glucose depression. Neither DDM alone in PBS, nor insulin 50 U/ml/kg alone showed any biological effect. The intravenously administered control, 0.5 U/ml/kg, demonstrated a significant reduction in blood glucose concentration (Fig. 2). This was observed as

a rapid blood glucose reduction with a concentration minimum reached after 1 h, followed by a gradual increase to normal levels. The time of glucose depression lasted for 4–5 h. The average dAUC under the curve was found to be 18.6 ± 4.44%$_*$h. The observed bioactivity of insulin coadministered ID together with DDM is shown in Fig. 3. All doses produced absorption of insulin as determined from blood glucose concentration depression. Based on dAUC, an apparent bioavailability of 0.84% for the dose of 50 U/ml/kg was estimated. This value decreased with an increase in the insulin dose. This is probably due to the fact that a larger portion of the insulin in the higher concentration is degraded, resulting in a lower bioavailability. The low bioavailabilities are in full agreement with earlier literature reports on the oral absorption of insulin and other peptides (Lee et al., 1991).

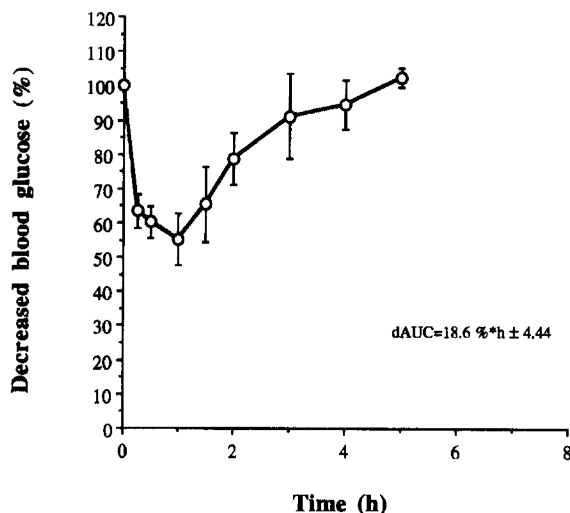

Fig. 2. Effect of intravenous administration of insulin on blood glucose levels in normal rats. Blood glucose depression determined as % of initial blood glucose concentration for each rat. Insulin dose was 0.5 U/kg in PBS (n = 4, ± S.D.).

3.2. Serum insulin levels

The success rate for diabetes induction in the rats was 95%. This was based on the blood glucose levels, which were 21.11 ± 2.8 mmole/l in a non-fasted state. The basal levels of insulin were measured to 35 μU/ml in control experiments. Intravenous administration of 0.25 U/ml/kg resulted in an immediate high insulin serum concentration (Fig. 4). The initial insulin concentration was 230 ± 12 μU/ml, and elimination of insulin from the blood was very rapid, characterized by a half-life of about 4 min. Basal serum insulin levels were reached 20 min after dosing. The AUC from time zero to 30 min was calculated to 31.2 ± 3.3 μU/ml$_*$h. Oral administration of 50 U/ml/kg insulin mixed alone did not result in any increase in the insulin concentrations. Fig. 5 shows serum insulin concentration after intraduodenal administration of 50 and 150 U/ml/kg insulin with DDM. The high dose of 150 U/ml/kg gave rise to a rapid increase in the serum insulin concentration. The concentration rose rapidly to peak levels of 257 μU/ml. Complete elimination took place over 20–30 min, with a half-life of about 4 min. Intraduodenal administration of insulin with DDM showed a minimal absorption over baseline values. Insulin absorption peaked at 116 μU/ml and was eliminated over a 20 min time period. AUC for 50 and 150 U/ml/kg were 28.6 ± 5.8 μU/ml$_*$h and 42.4 ± 11.5 μU/ml$_*$h, respectively.

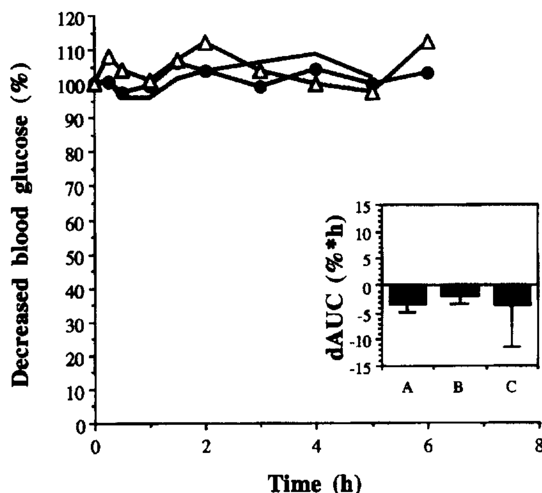

Fig. 1. Effect of intraduodenal controls on blood glucose levels in normal rats. Blood glucose depression determined as % of initial blood glucose concentration for each rat. Insert: Decreased areas under the blood glucose depression curves. Phosphate buffered saline 1 ml/kg (no symbol and (A) (n = 4, ± S.D.), DDM in PBS 1 ml/kg (circle and B) (n = 4, ± S.D.), native insulin 50 U/kg in PBS (triangle and C) (n = 4, ± S.D.).

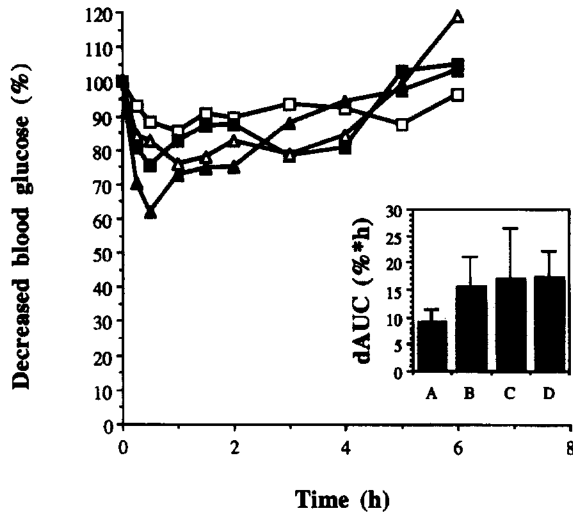

Fig. 3. Effect of intraduodenal administration of insulin with DDM in PBS on blood glucose levels in normal rats. Blood glucose depression determined as % of initial blood glucose concentration for each rat. Insert, S.D. = 13%: Decreased areas under the blood glucose depression curves, ± S.D. 25 U/kg (open square and A) (n = 4), 50 U/kg (closed square and B) (n = 6), 75 U/kg (open triangle and C) (n = 4), 150 U/kg (closed triangle and D) (n = 4).

In the oral absorption of any peptide from the intestine, it is desirable to have the lowest associated form of the peptide present at the site of absorption since absorption is size dependent (Loehry et al., 1973). The hydrodynamic radius of insulin in monomeric form is an estimated 12–13 Å (Bohidar and Geissler, 1984). As self-association progresses, the species size increases to about 19 Å for the dimeric state and about 30 Å for the hexameric state. If this is considered in relation to a paracellular absorption of insulin from the intestine, where pore sizes have been postulated to range between 7.1 and 16.0 Å by Hayashi et al. (1985), one can see the importance of keeping the molecular weight as low as possible. In vivo, it was necessary to use a higher complex ratio than that reported for in vitro stabilization to achieve insulin absorption (Hovgaard et al., 1996). This can be due to a competitive interaction of insulin monomer and other molecules in the lumen of the intestine with the stabilizing molecules. Moreover, an unknown dilution factor should always be taken into account in vivo. The bioavailabilities obtained from the two series of absorption experiments deviate by a factor of two. The bioavailability obtained from serum insulin measurements is roughly half that of the bioavailability obtained from blood glucose measurements. This discrepancy may be due to the basic differ-

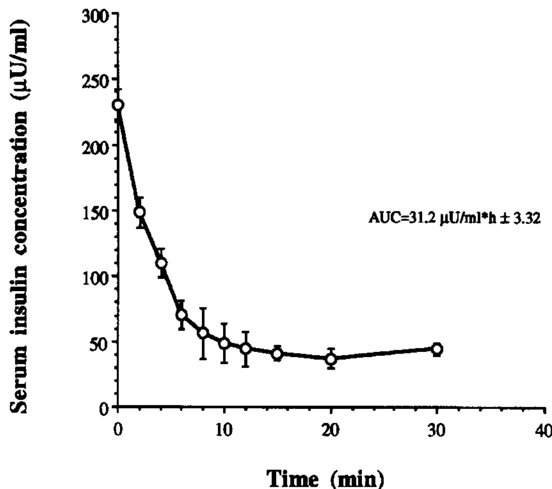

Fig. 4. Serum insulin concentrations after intravenous administration of insulin in streptozotocin diabetic rats. Insulin dose was 0.25 U/kg in PBS (n = 3, ± S.D.).

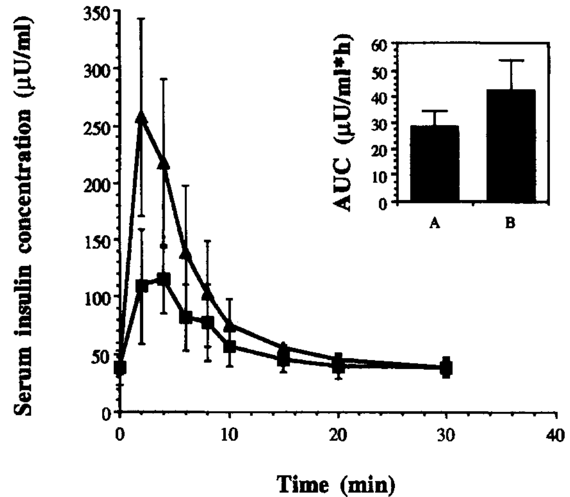

Fig. 5. Serum insulin concentrations after intraduodenal administration of insulin with DDM in PBS in streptozotocin diabetic rats. Insert: Areas under the serum insulin curves. 50 U/kg (square and A) (n = 4, ± S.D.), 150 U/kg (triangle and B) (n = 4, ± S.D.).

ences in the techniques. Insulin is absorbed from the intestinal tract and is transported directly to the liver (first pass effect). The liver accounts for nearly 50% reduction in the initial amount due to metabolism and receptor binding (Rubenstein et al., 1972). The insulin concentrations measured in this study were determined in systemic blood from the jugular vein. Therefore, the insulin concentration is lower than the actual absorbed amount. The measurement of blood glucose, however, should not be affected by the first pass metabolized fraction of absorbed insulin since the hepatic metabolism is a normal part of the blood glucose regulation.

In conclusion, it has shown that complex formation between insulin and alkylmaltoside is able to promote oral absorption. We have previously proposed that the uptake of insulin into micelles is a main mechanism for the enhanced stability (Hovgaard et al., 1996). Therefore, it is reasonable to believe that a reduction in molecular weight from hexamer to lower associated states of insulin could be a contributing factor in the enhanced absorption. Moreover, the higher thermodynamic activity at the site of absorption due to stabilization can increase the driving force and thereby increase the transport. The stabilization, however, only produced a bioavailability of insulin between 0.5 and 1%. Therefore, more studies are needed to optimize an oral insulin formulation.

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