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Effects of benzoic acid and its analogues on insulin and glucagon secretion in sheep

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Abstract

The effects of benzoic acid and its analogues on insulin and glucagon secretion were investigated in conscious sheep. Intravenous injections of benzoic acid increased plasma insulin and glucagon concentrations in a dose-dependent manner between 39–1250 μmol/kg, with ED_{50}s for increasing both hormones of about 625 μmol/kg. Various derivatives of benzoic acid (625 μmol/kg) were administered and structure-activity relationships were examined. A single carboxylic group was essential for stimulating insulin and glucagon secretion, since both hormone responses were abolished with compounds in which the carboxylic group was replaced by sulfonic or phosphoric groups, or in which another carboxylic element was introduced (phthalic acids). Most of the compounds which introduced other elements (amino and hydroxy groups, and halogens) onto the benzene ring had an altered stimulating activity. Thus the pancreatic endocrine system can recognize the chemical structure of benzoic acid and its derivatives in detail and induce insulin and glucagon secretion in sheep.

Keywords: Benzoic acid; Endocrine pancreas; Insulin; Glucagon; Ruminant; (Sheep)

1. Introduction

Benzoic acid, a mono-carboxylic acid with a benzene ring, is used as an antifungal drug or a food preservative (Harvey, 1985). In addition, the derivatives of this compound demonstrate pharmacological activity, with salicylic acid being used as an anodyne and antifebrile agent (Booth, 1982). Benzoic acid and its derivatives are known to enhance the action of local anesthetics, as evaluated by measuring the pain sensibility of human skin in vivo and the action potentials from the crayfish giant axon and the rat cervical vagus in vitro (Hiji et al., 1987). Apart from these general biological actions, it has been reported that benzoic acid stimulates amylase release from perifused fragments of the pancreas in sheep and goats (Katoh and Yajima, 1989). In a recent report from our laboratory, i.v. injection of benzoic acid affected pancreatic hormone secretion in sheep in vivo (Mineo et al., 1994).

The aim of the present study was to examine the effect of benzoic acid on the endocrine pancreatic response in further detail, and to determine structure-activity relationships using a variety of derivatives of benzoic acid. The changes in plasma insulin and glucagon concentrations after i.v. injection of benzoic acid and its analogues were compared in sheep in vivo.

2. Materials and methods

2.1. Animals and diets

Six Suffolk wethers aged 1–2 years and weighing 42–53 kg were used. They were housed in metabolic cages and were fed orchard grass hay (100 g) and lucerne pellets (1000 g) once daily at 19:00 h. Water was available freely. At least 2 months before experiments began, the sheep had their left common carotid artery chronically placed in a loop of skin by surgical

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operation under general anesthesia with pentobarbitone sodium (25 mg/kg). During the postoperative recovery period, they were trained to be accustomed to the experimental surroundings and procedure. At least 3 days before experimentation, a polyethylene catheter for infusion of drugs was inserted into the jugular vein through a hypodermic needle. Catheters were kept patent by flushing and filling with a 3.8% (w/v) sterile solution of trisodium citrate.

2.2. Experimental procedure

On the day of an experiment, feed and water were withdrawn. At least 2 h before experimentation, an indwelling needle was inserted into the carotid artery in a loop and connected to a catheter. This catheter was used for blood sampling and was filled with sterile solution of 3.8% (w/v) trisodium citrate. Benzoic acid and its analogues were dissolved in sterile water at a concentration of 1 or 2 M and the pH of these solutions was adjusted to 7.4 with NaOH or HCl. Benzoic acid was administered intravenously through the jugular catheter at the following seven doses: 39, 78, 156, 312, 625, 1250 and 2500 μmol/kg. Derivatives of benzoic acid were injected at a fixed dose of 625 μmol/kg, which was the ED_{50} of benzoic acid for the insulin and glucagon secretory responses. Each solution was injected into six sheep over a 1-min period. The timing of blood sampling was at -15, 0, 5, 10, 15, 30, 45, 60, 90 and 120 min relative to the time of injection. Arterial blood (4 ml) was taken by syringe and was immediately transferred into polyethylene test-tubes cooled in ice water until the end of the experiment. Each test-tube contained 1.2 mg of EDTA and 0.05 mmol of benzamidine (Wako Pure Chemical, Osaka, Japan) per 1 ml of blood. All sheep received i.v. injections of the seven doses of benzoic acid first, followed by injections of its analogues in a randomized manner with at least a 2-day interval between injections. Each sheep was injected with only one dose of benzoic acid or one derivative on any day. To minimize the influence of diurnal variation, all tests were performed between 10:00 and 13:00 h.

2.3. Tested compounds

Benzoic acid and its derivatives were purchased from Wako Pure Chemical (Osaka, Japan) or Tokyo Kasei Kogyo (Tokyo, Japan). Seventeen compounds were used in the present experiment.

2.4. Analyses

Plasma was separated by centrifugation at 4° C. The glucose concentration was determined by a glucose oxidase method (Kabasakalian et al., 1974) using 20 μl of plasma. The remaining plasma was stored at -20° C for insulin and glucagon assays. Plasma insulin was determined by a slight modification of the radioimmunoassay (RIA) described by Sasaki and Takahashi (1980). Guinea pig anti-bovine insulin serum (ICN, Israel) and ^{125}I-labelled porcine insulin (NEN, Boston, USA) were purchased. Bovine insulin (Sigma, St. Louis, USA) was used as a standard. Intra- and inter-assay coefficients of variation for the insulin determination were 5.8% ($n = 6$) and 8.6% ($n = 5$), respectively. Plasma glucagon was measured by RIA (Sasaki et al., 1982). The antiserum OAL-123, which is specific for the *C*-terminal portion of pancreatic glucagon, was used for this assay (Nishino et al., 1981). Crystalline bovine glucagon used as a standard (Calbiochem, California, USA) and ^{125}I-labelled porcine glucagon (NEN, Boston, USA) were purchased. Intra- and inter-assay coefficients of variation for the glucagon determination were 8.5% ($n = 6$) and 10.6% ($n = 5$), respectively.

2.5. Statistical analysis

All results are expressed as means $\pm$ S.E.M. The significance of differences between zero time and subsequent times were determined using Wilcoxon signed-rank test ($P < 0.05$). The hormone increment was calculated as the difference between the value at zero time and the peak value after the injection for each sheep. Statistical analysis for comparisons of hormone increments between 625 μmol/kg injection of benzoic acid (ED_{50} for both hormone responses) and its analogues was also performed by using the Wilcoxon signed-rank test ($P < 0.05$).

3. Results

3.1. Effect of benzoic acid on plasma insulin and glucagon concentration

Fig. 1 shows typical responses of plasma insulin, glucagon and glucose concentrations after benzoic acid injection at 625 μmol/kg. Plasma insulin and glucagon increased immediately after the injection of benzoic acid. Plasma glucose also increased and returned to pre-injection values. These patterns of pancreatic hormones and glucose responses occurred with all doses of benzoic acid. The dose-response relationships for the insulin and glucagon increments induced by seven doses of benzoic acid are shown in Fig. 2. Injection of benzoic acid increased plasma insulin and glucagon concentrations in a dose-dependent manner up to 1250 μmol/kg, but a larger dose (2500 μmol/kg) reduced both hormone responses. The ED_{50} of benzoic acid for increasing both insulin and glucagon concentrations in plasma was about 625 μmol/kg.

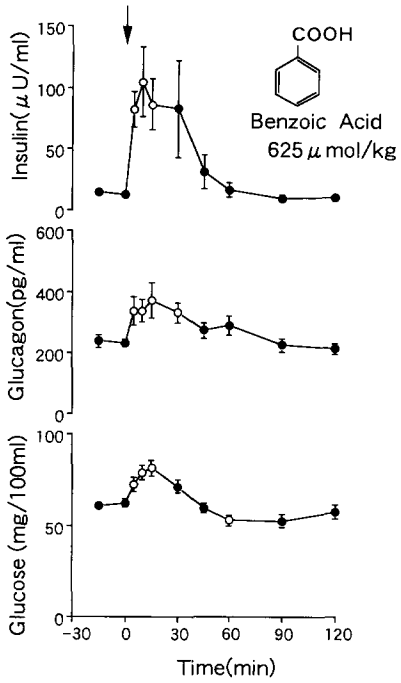

Fig. 1. Effects of benzoic acid on plasma insulin, glucagon and glucose concentrations. Six sheep each received an i.v. injection of benzoic acid at a dose of 625 μmol/kg (arrow). Values are means ± S.E.M. Open symbols indicate a significant difference from the value at zero time ($P < 0.05$).

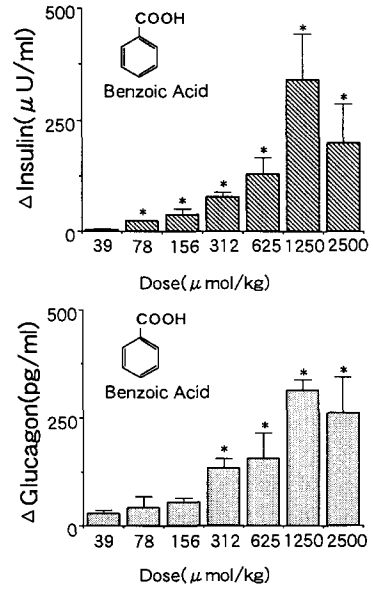

Fig. 2. Effects of benzoic acid on increments in plasma insulin and glucagon concentrations. Six sheep each received seven doses of benzoic acid (39–2500 μmol/kg). Hormone increments were calculated as the difference between the peak and basal values. Values are means ± S.E.M. Asterisks indicate that the increment in plasma hormone concentrations after injection of benzoic acid was significantly greater than the value at zero time ($P < 0.05$).

3.2. Structure-activity relationship

Requirement of carboxylic group

Intravenous injection of benzene-sulfonic acid or benzene-phosphonic acid (625 μmol/kg), in which the carboxylic group (-COOH) of benzoic acid is replaced by a sulfonic ($-SO_2OH$) or phosphonic group ($-PO(OH)_2$), did not affect plasma insulin, glucagon and glucose concentrations (data not shown). In addition, the effects of i.v. injections of *o*-, *m*- and *p*-phthalic acids (625 μmol/kg), which introduced a second carboxylic group onto the benzene ring at *o*-, *m*-, and *p*-position, were examined. No isomer had any effect on plasma insulin, glucagon and glucose concentrations (data not shown).

Modification of benzene ring

The increments in plasma insulin concentrations after injections of benzoic acid and its analogues at 625 μmol/kg are shown in Fig. 3. Most of the derivatives increased plasma insulin concentrations. However, the magnitude of this effect differed among the tested compounds. The introduction of an amino group ($-NH_2$) decreased the activity of benzoic acid in stimulating insulin secretion ($P < 0.05$). The introduction of a hydroxy group (-OH) at the *m*- and *p*-positions, but not at the *o*-position, decreased insulinotropic activity ($P < 0.05$). Introduction of chlorine (-Cl) into the benzene ring at any position did not affect the insulin-increasing response. Although introduction of bromine (-Br) at the *m*- and *p*-positions did not affect in-

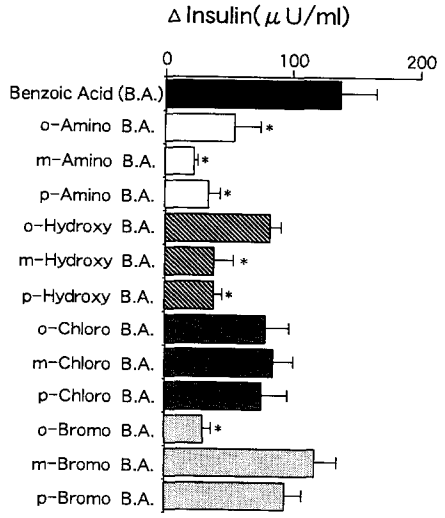

Fig. 3. Increments in plasma insulin concentration after injection of benzoic acid and its analogues. Hormone increments were calculated as the difference between the peak and basal values. Values are means ± S.E.M. Asterisks indicate that the increment in plasma hormone concentrations was significantly different to that after 625 μmol/kg injection of benzoic acid ($P < 0.05$).

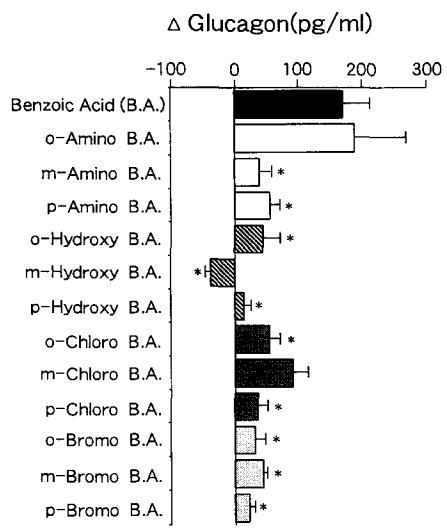

Fig. 4. Increments in plasma glucagon concentration after injection of benzoic acid and its analogues. Hormone increments were calculated as the difference between the peak and basal values. Values are means ± S.E.M. Asterisks indicate that the increment in plasma hormone concentrations was significantly different to that after 625 μmol/kg injection of benzoic acid ($P < 0.05$).

sulinotropic activity, *o*-bromo-benzoic acid showed a very much weaker effect on insulin secretion than benzoic acid did ($P < 0.05$). Fig. 4 shows glucagon increments after injections of benzoic acid and its derivatives. *o*-Amino- and *m*-chloro-benzoic acids were as effective as benzoic acid itself. However, the other derivatives had very much weaker effects on glucagon secretion than benzoic acid did ($P < 0.05$). *m*-Hydroxy-benzoic acid rather decreased the plasma glucagon concentration below the basal value ($P < 0.05$) (data for time course change not shown). Chemical modification of the benzene ring had greater effects on the ability to stimulate glucagon secretion compared with insulin secretion.

4. Discussion

It was clearly demonstrated in this experiment that i.v. injection of benzoic acid stimulates both insulin and glucagon secretion in sheep. The increments in plasma insulin and glucagon increased with the dose, up to a maximum response to both hormones which was obtained at 1250 μmol/kg benzoic acid. A larger dose (2500 μmol/kg) of benzoic acid tended to decrease the responses of both hormones. It was revealed that intravenous injection of 5000 μmol/kg NaCl solution used as a control (high tonic solution with large volume) did not affect plasma insulin and glucagon concentrations in sheep in vivo (Mineo et al., 1990b). From the determination of structure-activity relationships, a single carboxylic group combined with the benzene ring was absolutely required for stimulation of pancreatic hormone secretion. The introduction of amino or hydroxy groups, or halogens onto the benzene ring also affected the stimulating activity on insulin and glucagon secretion. Not only the introduction of a substituent, but also the position of insertion in the benzene ring had clear effects on hormone secretory activity. These results indicate that the endocrine pancreas in sheep can recognize the chemical structure of benzoic acid and its analogues in detail and induce insulin and glucagon secretion.

It has been reported that one of the characteristics of the pancreatic endocrine response in ruminants is that insulin and/or glucagon secretion is stimulated by i.v. injection of short-chain fatty acids in sheep (Manns and Boda, 1967; Horino et al., 1968; Trenkle, 1970; Bassett, 1972; Ambo et al., 1973; Mineo et al., 1990a,b), goats (Stern et al., 1970; De Jong, 1982) and cows (Horino et al., 1968). This short-chain fatty acid-induced insulin secretion has also been demonstrated in vitro in sheep (Hertelendy et al., 1968; Sasaki et al., 1977; Jordan and Phillips, 1978). Such responses to short-chain fatty acids do not occur in pigs, rats and rabbits (Horino et al., 1968). The patterns of hormone secretion after benzoic acid injection were similar to the response induced by short-chain fatty acids, in that both insulin and glucagon secretion were simultaneously stimulated, and their responses occurred over a short time course. In respect of their chemical structure, both compounds have a single carboxylic group and a number of hydrocarbon elements in the molecule.

Plasma glucose concentrations were also changed after the injection of benzoic acid. The changes in plasma glucose following i.v. injections of short-chain fatty acids in ruminants were also demonstrated in the previously cited reports. Since pancreatectomy completely abolished the changes in glucose after injection of short-chain fatty acids (Phillips et al., 1969), the changes in plasma glucose after short-chain fatty acid injection may have occurred as a result of the hyperglycemic effect of glucagon and the hypoglycemic effect of insulin. The same mechanism might be involved in the changes in plasma glucose following benzoic acid and its derivatives in this experiment.

It has been reported that a variety of mono-carboxylic acids (short-chain fatty acids, their analogues and benzoic acid) stimulate amylase release from perifused fragments of the pancreas in sheep and goats (Katoh and Yajima, 1989). From a determination of structure-activity relationships, a single carboxylic group was absolutely required in order to stimulate amylase release. A recent report from our laboratory revealed that a mono-carboxylic group in the structure of short-chain fatty acids was absolutely essential to stimulate insulin and glucagon secretion in sheep (Mineo et al.,

1994). Katoh and Yajima (1989) proposed the possibility that a specific receptor, which could recognize the chemical structure of short-chain fatty acids or their analogues (including benzoic acid), may exist on the surface of the exocrine cells of the pancreas in ruminants. It is possible that a specific receptor mechanism on the endocrine cells of the pancreas is involved in short-chain fatty acid and/or benzoic acid-induced insulin and glucagon secretion in sheep.

However, it has been reported that the local anesthetic action of procaine is enhanced by benzoic acid and its analogues, and that replacement of the carboxylic group by a sulfonic element abolishes this enhancement of procaine activity (Hiji et al., 1987). Some organic acids (including benzoic acid) were shown to affect the axonal membrane in crayfish and to accelerate procaine adsorption into the lipid bilayer (Ichikawa, 1987). In addition, *n*-butyric acid, a short-chain fatty acid, changes the fluidity of the cell membrane of colon cancer cells (Dibner et al., 1985). These results raise the possibility that benzoic acid changes the fluidity of the cell membrane, and thereby induces hormone secretion from the endocrine cells of the pancreas.

In conclusion, i.v. injection of benzoic acid and some of its derivaties stimulates both insulin and glucagon secretion in sheep in vivo. Determination of structure-activity relationships reveals that a single carboxylic group in the molecule is necessary to induce insulin and glucagon secretion. The characteristics of the hormone responses suggest that a similar mechanism to that by which i.v. injected short-chain fatty acids stimulate pancreatic hormone secretion in ruminants may play a role in this response.

In this experiment, it was clearly demonstrated that benzoic acid or its derivatives induced insulin and glucagon responses in peripheral plasma of sheep. However, the mechanism of organic acid (including benzoic acid)-induced pancreatic hormone secretion is not known in detail. Thus, further experiments, using in vitro preparations, are needed to clarify the mechanism for recognition of the chemical structure of benzoic acid and its analogues and the stimulus-secretion coupling for benzoic acid-induced hormone release from the endocrine cells of the pancreas in sheep.

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