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Autonomic Nervous System Mediation of the Pancreatic Polypeptide Response to Insulin-Induced Hypoglycemia in Conscious Rats*

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ABSTRACT. To investigate the neural regulation of pancreatic polypeptide (PP) secretion during hypoglycemia in the rat, insulin was administered to chronically cannulated rats, and plasma PP responses were compared between saline-treated animals and animals pretreated with a ganglionic blocking agent (hexamethonium), a muscarinic antagonist (atropine), combined α - and β -adrenergic receptor blockade (propranolol + tolazoline), or combined adrenergic blockade + atropine. PP was measured using a new RIA which selectively detects PP in rat plasma. In control rats ($n = 10$), plasma PP increased from a baseline level of 30 ± 3 pg/ml to 271 ± 41 pg/ml during hypoglycemia (plasma glucose = 29 ± 2 mg/dl) (Δ PP = $+241 \pm 42$ pg/ml, $P < 0.0005$), demonstrating that in rats, as in other species, insulin-induced hypoglycemia is a potent stimulus for PP release. PP only increased by 31 ± 10 pg/ml during similar hypoglycemia in 7 hexamethonium-treated rats ($P < 0.01$ vs. control animals). Thus, at least 90% of the PP response to

hypoglycemia is neurally mediated. The plasma PP response to hypoglycemia was $+85 \pm 24$ pg/ml in atropine-treated rats ($P < 0.01$ vs. control rats), suggesting that approximately 65% of the PP response is mediated via muscarinic acetylcholine receptors on the islet F cell. The PP response to hypoglycemia in rats with combined adrenergic blockade ($\Delta = +168 \pm 32$ pg/ml) was slightly, but not significantly smaller than that in control rats. The combination of combined blockade + atropine resulted in a PP response ($\Delta = +26 \pm 7$ pg/ml) to hypoglycemia that was similar to that in hexamethonium-treated rats ($P < 0.01$ vs. control rats). These results suggest: 1) The PP response to hypoglycemia is predominately the result of muscarinic, cholinergic activation. 2) There is a minor adrenergic contribution to the response. 3) The plasma PP response may be useful as an index of autonomic neural input to the islet during hypoglycemia. (*Endocrinology* 130: 2225-2229, 1992)

THE SECRETION of the islet hormone, pancreatic polypeptide (PP), is extremely sensitive to activation of vagal input to the pancreas. Electrical stimulation of the vagus nerves or the administration of cholinergic agonists potently stimulates PP secretion via an atropine-sensitive mechanism (1-4). Vagal control of PP release is also mediated by autonomic neurotransmission between pre- and postganglionic parasympathetic nerves because the ganglionic blocking agent, hexamethonium, abolishes the PP response to vagal nerve stimulation (3). It is thought that vagal, parasympathetic input to the pancreas is activated during central neuroglucopenia be-

cause plasma PP increases during glucopenic stress produced by insulin-induced hypoglycemia (5) or by administration of 2-deoxy-D-glucose (6), and this response is largely eliminated by vagotomy (7, 8).

The peripheral neural pathway and neurochemical mediators of glucopenia-induced PP secretion have been characterized in several species (see reviews in Refs. 9 and 10). In humans, and in dogs, the PP response to neuroglucopenia is nearly eliminated by atropine, demonstrating that the response is almost entirely the result of muscarinic, cholinergic stimulation (4, 5). In addition, since graded doses of acetylcholine or graded degrees of electrical vagal nerve stimulation produce proportional increases of PP secretion (5), it has been suggested that plasma PP levels may be used as a quantitative rather than simply as a qualitative index of parasympathetic input to the pancreas. Thus, measurements of plasma PP, and PP responses to neuroglucopenia, have been used by numerous investigators to assess the effectiveness of therapeutic vagotomy (11), as a marker for autonomic neuropathy (12, 13), and as an index of the para-

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sympathetic input to both the endocrine and exocrine pancreas (6, 14, 15). However, in experiments conducted in calves, electrical stimulation of sympathetic nerves increases plasma PP levels, and this increase is attenuated by the administration of adrenergic antagonists (16). Therefore, plasma PP responses to neuroglucopenia may not be solely mediated by parasympathetic neural activation in all species, since neuroglucopenia is also known to produce sympathoadrenal activation (17, 18).

Investigation into the regulation of PP secretion in rodents has been prevented by two major problems. First, rat PP differs from human, canine, bovine, and porcine PP by eight amino acids (19). Therefore, antisera that are effective for measuring PP in these species are not necessarily effective for measuring PP in rat plasma. Second, antisera directed against the C-terminal hexapeptide of PP, which does not vary between rats and other species, have not proved useful because these antisera cross-react with neuropeptide-Y (NPY) and peptide-YY (PYY) which are homologous with rat PP at four of the six amino acid residues. Thus, the neural regulation of PP secretion, which has been well characterized in other species, has not been established in the rat. We have recently developed and validated a sensitive assay for rat PP which does not cross-react with NPY or PYY (20).

Therefore, to determine whether plasma PP levels are increased during neuroglucopenia in conscious rats, plasma PP levels were measured before and during insulin-induced hypoglycemia. To test whether hypoglycemia-induced PP release is neurally mediated, rats were pretreated with the ganglionic blocker, hexamethonium, which impairs neurotransmission across parasympathetic and sympathoadrenal nicotinic ganglia (21). To determine the respective contributions of muscarinic, cholinergic, or adrenergic receptor activation to hypoglycemia-induced increases of plasma PP, atropine or a combination of α - and β -adrenergic antagonists was administered before the induction of hypoglycemia. Finally, to determine the combined contribution of cholinergic and adrenergic activation, another group of rats received both atropine and adrenergic blockers.

Materials and Methods

Animals and cannula implantation

Adult male Sprague-Dawley rats (390–510 g, Bantin and Kingman, Fremont, CA) were used for these studies. Rats were individually housed and fed a stock diet (Ralston Purina) and water *ad libitum*. The light/dark cycle was 12 h on and 12 h off, with lights on at 0600 h. In order to access mixed venous blood, each animal had a silicon jugular cannula (id, 0.3 mm; od, 0.6 mm, American Scientific, McGraw Park, IL) implanted in the right atrium via the right jugular vein under pentobar-

bital anesthesia (65–80 mg/kg ip). The cannula was attached to a 2 cm piece of 20 gauge, L-shaped stainless steel tubing and routed sc to the top of the head. The steel tube with the attached cannula was then fixed to the skull using three small steel screws and dental cement. The cannulas were flushed with heparinized saline (50 U/ml) and capped when not in use. After the cannula implantation, a minimum of 72 h was allowed for recovery before experiments were conducted.

Hypoglycemia protocol

All animals were fasted overnight (~18 h) before the experiments. Experiments were conducted between 1100 and 1600 h. Either saline, hexamethonium bromide (10 mg/kg), or atropine methyl bromide (Sigma Chemical Co., St. Louis, MO) (10 mg/kg) were administered iv. Atropine methyl bromide was used rather than atropine sulfate because atropine methyl bromide does not readily cross the blood-brain barrier (21), and thus should not impair central nervous system muscarinic, cholinergic neurotransmission. A fourth group received a combination of a β -adrenergic blocker, propranolol HCl (1 mg/kg), Inderal, Ayerst Laboratories, New York, NY) and an α -adrenergic blocker, tolazoline HCl (10 mg/kg) (Priscoline, Ciba-Geigy, Summit, NJ). Lastly, both muscarinic (atropine) and combined adrenergic blockade (propranolol + tolazoline) were induced in a fifth group of rats. Fifteen minutes after the administration of autonomic blockers baseline samples were drawn from the jugular cannula. Then 1.0–2.0 U/kg of regular porcine insulin (Squibb-Novo Inc., Bagsvaerd, Denmark) was administered iv as a bolus. A second blood sample was drawn 30 min after the insulin injection. After each blood sample was drawn, an equal volume of heparinized blood (20 U/ml) from donor rats was replaced via the jugular cannula.

Assays and data analysis

Blood samples for glucose and PP determination were placed in tubes containing EDTA. All samples were kept on ice until centrifugation (20 min at 4°C). The plasma was then decanted and frozen at –20°C until assayed.

Plasma glucose was assayed by the glucose oxidase method with a glucose analyzer (Beckman Instruments, Fullerton, CA). Plasma immunoreactive PP was measured radioimmunologically in unextracted plasma with a guinea pig-derived antisera. PP levels in rat plasma were determined by a RIA previously described (20). The procedure consists of a step-wise addition of antibody and a labeled ligand in a disequilibrium incubation. Guinea pig antirat-PP serum (100 μ l) obtained from Linco Research, Inc. (St. Louis, MO) and plasma samples (100 μ l) were incubated for 72 h. [125 I]rat-PP tracer (100 μ l) was then added, and the tubes were incubated an additional 24 h. The free 125 I was separated from the bound 125 I by a second antibody precipitation method using goat anti-guinea pig immunoglobulin G serum and normal guinea pig serum carrier (20). PP concentrations in both standard and unknown samples were analyzed by counting the pellet in an automated γ -counter (Apex, Micromedex Systems, Inc., Horsham, PA). The calculations were performed with a data reduction system using log/logit transformation.

The changes of plasma glucose and PP were calculated by subtracting the 0 min baseline value from the 30 min value

after the injection of insulin. The data are expressed as mean $\pm$ SE. Statistical comparisons of means within a group were made with a paired *t* test. For statistical comparisons of means of different groups, analysis of variance was performed followed by Dunnett's posttest.

Results

Plasma glucose before and after insulin injection

In control rats ($n = 10$), baseline plasma glucose averaged 100 ± 3 mg/dl and fell to 29 ± 2 mg/dl 30 min after insulin administration ($\Delta = -71 \pm 2$ mg/dl) (Fig. 1). Neither the degree of hypoglycemia nor the glucose decrement produced by insulin differed significantly among the five groups of animals. For example, in the hexamethonium-treated rats, baseline plasma glucose was 97 ± 3 mg/dl ($n = 7$) and decreased to 26 ± 2 mg/dl, 30 min after insulin injection ($\Delta = -71 \pm 4$ mg/dl).

Plasma PP before and during insulin-induced hypoglycemia

Baseline plasma PP in control rats was 30 ± 3 pg/ml. Plasma PP increased by more than 7-fold during insulin-induced hypoglycemia; to 271 ± 41 pg/ml ($\Delta = +241 \pm 42$ pg/ml, $P < 0.0005$) (Fig. 2). Plasma PP in hexamethonium-treated rats averaged 32 ± 4 pg/ml before insulin injection. There was a small but significant increase of PP during hypoglycemia ($\Delta = +31 \pm 10$ pg/ml, $P < 0.025$). This response was significantly smaller than the response in control rats ($P < 0.01$). In atropine-treated rats, baseline PP averaged 27 ± 2 pg/ml. In six out of seven atropinized animals the PP was ≤ 25 pg/ml, which is the level of detection for this assay. Plasma PP was increased significantly during hypoglycemia in atropinized rats; by $+85 \pm 24$ pg/ml ($P < 0.01$). However, this response was reduced by approximately 65% and was

FIG. 1. Plasma glucose levels before and after iv insulin injection in control, atropine-treated, hexamethonium-treated, propranolol + tolazoline-treated, and propranolol + tolazoline + atropine-treated conscious rats.

FIG. 2. Plasma PP levels before and during insulin-induced hypoglycemia in control, atropine-treated, and hexamethonium-treated conscious rats.

FIG. 3. Plasma PP levels before and during insulin-induced hypoglycemia in control, propranolol + tolazoline-treated, and (propranolol + tolazoline) + atropine-treated conscious rats.

significantly smaller than the response in control rats ($P < 0.01$). Plasma PP in rats treated with both α - and β -adrenergic antagonists increased from 51 ± 8 pg/ml to 219 ± 31 pg/ml during hypoglycemia ($\Delta = +168 \pm 32$ pg/ml, $P < 0.0025$) (Fig. 3). In rats that received both atropine and combined adrenergic blockade, baseline plasma PP was 39 ± 6 pg/ml and increased to 66 ± 7 pg/ml ($\Delta = +26 \pm 7$ pg/ml, $P < 0.005$). This response was significantly reduced compared to that in the control rats ($P < 0.01$).

Discussion

In this study, we have demonstrated that plasma PP levels increase during hypoglycemia in conscious rats. To our knowledge, this is the first demonstration of increased PP release during central glucopenia in this species. Because PP was only measured at one time point during hypoglycemia, the glucose threshold for PP release in the rat was not determined. This study was made possible by the development of an assay for PP in rat plasma which uses antiserum that is both sensitive and specific for rat PP (20). Earlier attempts to measure plasma PP in rats have utilized antisera directed against the C-terminal hexapeptide of PP (22, 23). The baseline levels of PP reported in these two studies were much

larger (>200 pg/ml) than in the present study (~30 pg/ml). It is likely that these higher levels, in fact, represent a cross-reactivity with circulating neuropeptide-Y (NPY), which has significant homology with the C-terminal hexapeptide (19). Plasma NPY levels are known to be high in rats, and are thought to be derived from platelets (24). Measurements of PP levels with the hexapeptide-directed antisera did not demonstrate a significant increase of plasma PP during insulin-induced hypoglycemia in rats (22, 23). However, these studies were performed in rats anesthetized with pentobarbital, an anesthetic which is known to markedly inhibit the PP response to central glucopenic stress (4, 6).

Pretreatment with the nicotinic acetylcholine antagonist hexamethonium, inhibited the PP response to insulin-induced hypoglycemia by nearly 90%, demonstrating that the majority of the plasma PP response to insulin-induced hypoglycemia is neurally mediated and that nicotinic neurotransmission across autonomic ganglia is involved in the neural pathway mediating the response. Hexamethonium has been demonstrated to inhibit the PP response to neuroglucopenia induced by 2-deoxy-D-glucose in dogs (25). The small portion of the response not blocked by hexamethonium could potentially result from a direct effect of low glucose levels at F cell or to stimulation by unidentified humoral factors released during hypoglycemia.

Pretreatment with atropine reduced the PP response by approximately 65% suggesting that, as in other species (4, 5), PP release during glucopenic stress is mediated, at least in part, by muscarinic acetylcholine receptors on the pancreatic F cell. However, in dogs and in humans, the PP response to neuroglucopenia is nearly abolished by atropine (4, 5) suggesting that potential species-related differences are present in the amount of cholinergic contribution to the response.

The PP response to insulin-induced hypoglycemia was smaller in rats treated with α - and β -adrenergic antagonists than in control rats, but this modest decrease did not achieve statistical significance. However, the addition of combined adrenergic blockade to muscarinic blockade with atropine resulted in a plasma PP response that was equivalent to that in hexamethonium-treated animals. Thus, it appears that, in rats, the PP response to hypoglycemia may be partially mediated by adrenergic, in addition to cholinergic mechanisms. This result contrasts with data obtained from experiments in other species in which the PP response to insulin-induced hypoglycemia is almost entirely the result of muscarinic, cholinergic activation (4, 5). In humans, α -adrenergic agonism inhibits PP secretion and β -adrenergic agonism stimulates PP secretion (26). However, the dominant catecholamines in plasma, epinephrine and norepinephrine, have little net effect on either basal (26, 27) or

insulin hypoglycemia-induced PP release (28), perhaps because of the opposing α - and β -adrenergic effects of these mixed agonists. Although, the effects of adrenergic antagonists on the PP response to hypoglycemia have not been examined in other species, it has been previously shown that electrical stimulation sympathetic (splanchnic) nerves increases plasma PP in calves, and this increase is attenuated by adrenergic antagonists (16). In addition, there is evidence for adrenergic regulation of PP secretion in avian species (29, 30). Therefore, in some species, there is potential for adrenergic mediation of PP secretion.

In summary, we have demonstrated a neurally mediated increase of plasma PP during insulin-induced hypoglycemia in the rat. Similar to other species, most of this increase is mediated by muscarinic acetylcholine receptors on the pancreatic F cell. However, in contrast to other species, a small portion of the PP response to hypoglycemia may be adrenergically mediated. Thus, the atropine-sensitive component of plasma PP levels and the plasma PP response to insulin-induced hypoglycemia may be useful as an index of the parasympathetic, cholinergic input to the pancreas in rats provided that the potential for mediation by other factors is carefully considered and investigated.

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