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Glucagon secretory response to hypoglycaemia, adrenaline and carbachol in streptozotocin-diabetic rats

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Glucagon response to insulin-induced hypoglycaemia is impaired in diabetes, but the mechanism is not established. Pancreatic A cell hyporesponsiveness to adrenergic or cholinergic stimulation could contribute to the impairment. We therefore compared the plasma glucagon responses to intravenous infusion of adrenaline (1200 ng kg^{-1} min^{-1} for 20 min) or to intravenous injection of the cholinergic agonist carbachol (50 μg kg^{-1}) in chloral hydrate-anaesthetized rats made diabetic with the use of streptozotocin (80 mg kg^{-1} subcutaneously) 6 weeks before and in anaesthetized control rats. Insulin was infused intravenously to reduce plasma glucose levels to below 1.8 mmol L^{-1}. As expected, the plasma glucagon response was reduced by $\sim 45\%$ in streptozotocin-diabetic rats compared with controls ($P = 0.045$). During adrenaline infusion, plasma glucagon levels increased by 277 ± 92 pg mL^{-1} in controls ($P = 0.009$) and by 570 ± 137 pg mL^{-1} in the diabetic rats ($P = 0.002$). Thus, the plasma glucagon response to adrenaline was approximately doubled in the diabetic rats ($P = 0.045$). Following carbachol injection, plasma glucagon levels were raised by 1211 ± 208 pg mL^{-1} ($P < 0.001$) in controls but only by 555 ± 242 pg mL^{-1} in the diabetic rats ($P = 0.049$). Thus, the plasma glucagon response to carbachol was impaired by $\sim 58\%$ in the diabetic rats ($P = 0.028$). We conclude that carbachol-stimulated glucagon secretion is impaired concomitantly with the impaired glucagon response to hypoglycaemia in streptozotocin-diabetic rats, whereas adrenaline-induced glucagon secretion is exaggerated. We suggest that a reduced pancreatic A cell responsiveness to cholinergic stimulation could contribute to the impairment of the glucagon response to insulin-induced hypoglycaemia in diabetes.

Key words: adrenaline, cholinergic, diabetes, glucagon secretion, hypoglycaemia, rats.

Increased glucagon secretion during hypoglycaemia is a primary factor responsible for the restoration of plasma glucose levels following insulin administration (Cryer 1981). Insulin-induced hypoglycaemia activates both parasympathetic (Schwartz *et al.* 1978, Havel *et al.*

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1992a) and sympathetic (Havel *et al.* 1992b) nerves innervating the pancreas and in addition produces a neurally mediated (Cantu *et al.* 1963) release of adrenaline from the adrenal medulla (Cannon *et al.* 1924, Garber *et al.* 1978), which reaches the pancreas via its arterial blood supply. Activation of these three neural inputs to the pancreas involves independent neural processes, each of which can stimulate glucagon secretion (Gerich *et al.* 1974, Ahrén *et al.* 1986, 1987). Furthermore, autonomic activation has been demonstrated to mediate the majority of hypoglycaemia-induced glucagon secretion in rats (Borg *et al.* 1994, Havel *et al.* 1994), in mice (Havel *et al.* 1993) and in dogs (Briggers *et al.* 1989, Havel *et al.* 1991). This topic has recently been extensively reviewed (Havel & Taborsky 1989, 1994).

The glucagon secretory response to hypoglycaemia is impaired in both type 1 and type 2 diabetes (Gerich *et al.* 1973, Benson *et al.* 1977, Reynold *et al.* 1977, Hilsted *et al.* 1982, Meneilly *et al.* 1994), and a similar impairment has also been demonstrated in experimental diabetes in the rat (Patel 1983a, b, 1984a, b, Hertelendy *et al.*1992, Ishida *et al.* 1993, Powell *et al.* 1993). The underlying mechanisms responsible for the impaired hypoglycaemia-induced glucagon secretion are poorly understood, although autonomic neuropathy (Maher *et al.* 1977) and hypoglycaemia-associated autonomic failure (Dagago-Jack *et al.* 1993) have been implicated in clinical situations. Previous studies have shown that the adrenaline response to hypoglycaemia is impaired (Patel 1983a, Powell *et al.* 1993), and that the glucagon secretory responses to vagal nerve activation (Hertelendy *et al.* 1992) and sympathetic nerve stimulation (Kurose *et al.* 1992) are impaired in diabetic rats, suggesting that impaired hypoglycaemia-induced glucagon secretion in diabetes could result from impaired sympathoadrenal or cholinergic activation or neurotransmission. However, reduced A-cell secretory responses to direct stimulation by adrenaline or to the cholinergic neurotransmitter, acetylcholine, could also contribute to impaired glucagon secretion during hypoglycaemia.

To investigate this possibility, we examined the glucagon secretory responses to insulin-induced hypoglycaemia and to direct stimulation with adrenaline or the cholinergic agonist, carbachol, in streptozotocin diabetes.

METHODS

Animals. Adult male Sprague–Dawley rats (350–400 g; Charles River Laboratory) were used. The 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 06.00 h. The animal preparation and experimental protocols were approved by the institutional animal use and care committee, and were conducted in accordance with the National Institutes of Health Guide for the Use and Care of Laboratory Animals.

Induction of diabetes. Streptozotocin (Sigma Chemical Co., St Louis, MO) was freshly prepared in ice-cold 0.05 mol L^{-1} citrate buffer, pH 4.5, and injected subcutaneously at the dose level of 80 mg kg^{-1}. Control rats were injected with ice-cold 0.05 mol L^{-1} citrate buffer alone. All streptozotocin-treated rats exhibited polydipsia, hyperphagia and elevated blood glucose levels (Table 1). The experiments were performed after 6 weeks.

Hypoglycaemia experiments. Rats were fasted overnight with free access to water. They were anaesthetized with chloral hydrate (350 mg kg^{-1} intraperitoneally) and polyethylene cannulae were inserted into a femoral vein (for drug administration) and a jugular vein (for blood sampling). Chloral hydrate was then continuously infused at 25 mg kg^{-1} min^{-1}. Chloral hydrate anaesthesia was chosen because we have previously demonstrated that neither parasympathetic nor sympathetic nerve responses to hypoglycaemia are affected by this agent, as opposed to the marked suppression of autonomic activation produced by pentobarbital anaesthesia (unpublished). Rats were allowed to stabilize for 30 min after surgery. A baseline blood sample was taken and its plasma glucose value immediately assessed, then a bolus

Table 1. Body weight, food and water intake, and baseline plasma glucose levels in control and streptozotocin-diabetic rats

Treatment	Control rats	Diabetic rats
Body wt (g)		
Initial	364±8	375±7
Final	521±11	351±5
Change	157±12	−24±8*
Intake		
Food (g day^{-1})	32±1	70±2*
Water (mL day^{-1})	61±4	291±11*
Plasma glucose (mmol L^{-1})	7.8±0.1	26.2±0.5*

Values are means±SE. Asterisk indicates a probability of random difference of $P < 0.001$ between the groups.

injection of regular porcine insulin (Novo Inc., Bagsvaerd, Denmark) was given according to the formula $[2.0 + (\text{plasma glucose level in mg dL}^{-1} - 100) \times 0.02]$ U kg^{-1}; followed by, in diabetic rats, a continuous intravenous infusion of insulin at the dose rate of 5 U kg^{-1} h^{-1} (Patel 1984a). A new blood sample was taken at 30 min, and then, in diabetic rats, plasma glucose was determined every 30 min. When the plasma glucose level had dropped to 1.8 mmol L^{-1}, a final blood sample was taken.

Experiments with adrenaline and carbachol. Following anaesthesia, insertion of catheters as above and a 30 min stabilization period, a blood sample was taken and then adrenaline (Sigma) was infused intravenously at the dose level of 1200 ng kg^{-1} min^{-1} for 20 min. Carbachol (Sigma) was injected rapidly intravenously, 40 min after the adrenaline infusion had been discontinued, at the dose level of 50 μg kg^{-1}. Blood samples were taken after 20 min of adrenaline infusion and immediately before and at 5 min after carbachol injection.

Assays. Blood samples for glucose determination were placed in tubes containing EDTA, whereas blood samples for glucagon determination were placed in tubes containing EDTA and aprotinin (Sigma). All samples were kept on ice until centrifugation at 4 °C for 20 min. The plasma was then decanted and frozen at −20 °C until assayed. Plasma glucose was analysed by the glucose oxidase method with a glucose analyser (Beckman Instruments, Fullerton, CA). Plasma immunoreactive glucagon was measured radioimmunochemically with an antibody with high specificity for the COOH-terminal portion of the glucagon molecule and therefore specific for pancreatic glucagon, using antisera and reagents supplied by Linco Research, St Louis, MO. Cross reactivity to gastrointestinal glucagon-like material (oxyntomodulin) is less than 0.1%.

Statistics. Data are presented as mean ± SE; either as absolute values or as changes in values from the value before the respective stimulations. The data were not expressed as percent increase, since there was no significant correlation between baseline plasma glucagon values and the glucagon responses to any of the stimulations. Statistical comparisons of means within a group were performed with a paired *t*-test, whereas comparisons of means of different groups of rats were performed with the Mann–Whitney *U*-test.

RESULTS

Insulin induced hypoglycaemia

Plasma glucose. Plasma glucose levels at the time of experiments, i.e. at 6 weeks after streptozotocin injection, were 7.8 ± 0.1 mmol L^{-1} in control ($n = 15$) and 26.2 ± 0.5 mmol L^{-1} in diabetic rats ($n = 12$; $P < 0.001$). At 30 min after insulin administration, plasma glucose levels were 1.7 ± 0.1 mmol L^{-1} in control and 15.8 ± 1.5 mmol L^{-1} in diabetic rats. At the time of the final blood sampling in diabetic rats, plasma glucose levels were 1.5 ± 0.1 mmol L^{-1}. This blood sample was taken 202 ± 21 min after insulin administration.

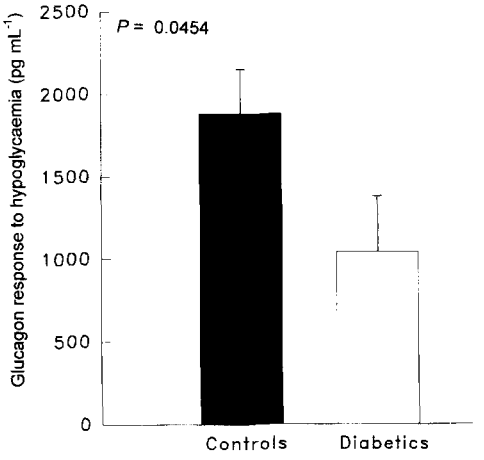

Fig. 1. The increase of plasma glucagon levels above baseline in response to insulin-induced hypoglycaemia in control ($n = 15$) and streptozotocin-diabetic rats ($n = 12$) at 6 weeks after induction of diabetes. Means ± SE are shown.

Plasma glucagon. Baseline plasma glucagon levels were 292 ± 60 pg mL^{-1} in control and 153 ± 14 pg mL^{-1} in diabetic rats (n.s.; $P = 0.180$). At 30 min after insulin administration, plasma glucagon levels were increased to 2175 ± 282 pg mL^{-1}, i.e. by 1883 ± 268 pg mL^{-1} ($P < 0.001$) in controls, whereas the plasma glucagon levels in the diabetics were unchanged at 170 ± 36 pg mL^{-1} (n.s.; $P = 0.868$). At the final blood sample in the diabetic rats, i.e. at the equivalent low plasma glucose level as in the controls, plasma glucagon levels were 1195 ± 376 pg mL^{-1}, i.e. they were increased by 1042 ± 336 pg mL^{-1} ($P = 0.019$). Thus, the increase in plasma glucagon levels during the insulin-induced hypoglycaemia was impaired by ~ 45% in the diabetics ($P = 0.0454$; Fig. 1).

Adrenaline infusion

Plasma glucose. Baseline plasma glucose levels were 10.2 ± 0.6 mmol L^{-1} in controls ($n = 16$)

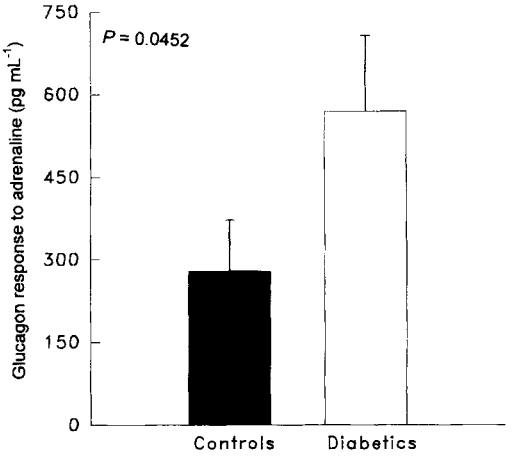

Fig. 2. The increase of plasma glucagon levels above baseline in response to 20 min intravenous infusion of adrenaline (1200 ng kg^{-1} min^{-1}) in control rats ($n = 16$) and in streptozotocin-diabetic rats ($n = 10$) at 6 weeks after induction of diabetes. Means ± SE are shown.

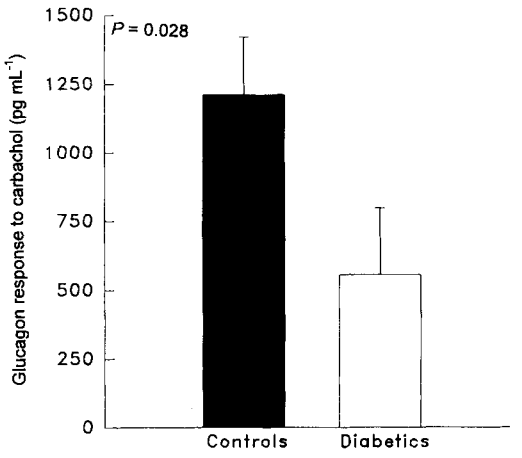

Fig. 3. The increase of plasma glucagon levels above baseline in response to a rapid intravenous injection of the cholinergic agonist carbachol (50 μg kg^{-1}) in control rats ($n = 13$) and in streptozotocin-diabetic rats ($n = 7$) at 6 weeks after induction of diabetes. Means ± SE are shown.

and 27.6 ± 1.3 mmol L^{-1} in diabetics ($n = 10$), and were not significantly altered by the 20 min adrenaline infusion (10.7 ± 0.8 mmol L^{-1}; $\Delta = +0.5 \pm 0.6$ mmol L^{-1}; n.s.; $P = 0.492$) in controls and 27.8 ± 1.3 mmol L^{-1} ($\Delta = +0.2 \pm 0.9$ mmol L^{-1}; n.s.; $P = 0.747$) in diabetics, respectively.

Plasma glucagon. During adrenaline infusion, plasma glucagon levels increased from 225 ± 33 to 504 ± 96 pg mL^{-1}, i.e. by 279 ± 92 pg mL^{-1} in controls ($P = 0.009$) and from 142 ± 34 to 712 ± 135 pg mL^{-1}, i.e. by 570 ± 137 pg mL^{-1} in diabetics ($P = 0.002$). Thus, the plasma glucagon response to adrenaline was approximately doubled in the diabetic rats ($P = 0.0452$; Fig. 2).

Carbachol injection

Plasma glucose. Plasma glucose levels were slightly reduced at 5 min after the rapid injection of carbachol from 11.0 ± 0.8 to 9.5 ± 1.1 mmol L^{-1} ($\Delta = -1.5 \pm 0.4$ mmol L^{-1}; $P = 0.036$) in the controls ($n = 13$), whereas they were not significantly altered in the diabetics ($n = 7$), averaging 29.4 ± 2.0 mmol L^{-1} before carbachol injection and 27.3 ± 2.6 mmol L^{-1} at 5 min after ($\Delta = -2.1 \pm 1.6$ mmol L^{-1}; n.s.; $P = 0.252$).

Plasma glucagon. Plasma glucagon levels increased from 414 ± 102 to 1625 ± 243 pg mL^{-1} at 5 min after carbachol injection, i.e. by 1211 ± 208 pg mL^{-1} ($P < 0.001$) in controls, and from 205 ± 28 to 760 ± 263 pg mL^{-1} at 5 min after carbachol injection in diabetic rats, i.e. by 555 ± 242 pg mL^{-1} ($P = 0.049$). Thus, the plasma glucagon response to carbachol was impaired by ~ 58% in the diabetic rats ($P = 0.028$; Fig. 3).

DISCUSSION

In this study, the plasma glucagon response to insulin-induced hypoglycaemia was impaired by ~ 45% in streptozotocin-diabetic rats at 6 weeks after induction of the diabetes. The glucagon response in the two groups of rats was determined from the plasma glucagon levels obtained when severe hypoglycaemia had evolved, which occurred later in the diabetic rats than in the control rats. However, it has previously been established that it is primarily the absolute glucose level rather than the rate of fall of glucose that determines the magnitude of the glucagon response to hypoglycaemia (Amiel *et al.* 1987). Previously, Patel has demonstrated a marked (> 90%) impairment of the glucagon response to hypoglycaemia at 12 weeks after

streptozotocin (Patel 1983a), whereas a normal glucagon response is seen at 10–15 days after streptozotocin (Patel 1984a). Taken together, the results suggest a gradual development of the impaired glucagon response in streptozotocin-diabetic rats. Similarly, a defect in the glucagon response to hypoglycaemia has been demonstrated in spontaneously diabetic rats (Powell *et al.* 1993) and in dogs (Havel *et al.* 1995) and in both type 1 and type 2 diabetic humans (Gerich *et al.* 1973, Benson *et al.* 1977, Reynold *et al.* 1977, Hilsted *et al.* 1982, Meneilly *et al.* 1994). Therefore, impairment of hypoglycaemia-induced glucagon secretion seems to be a general phenomenon in diabetes. The mechanisms underlying this impairment are, however, still poorly understood.

Previous studies in streptozotocin-diabetic rats have suggested several different mechanisms which could contribute to the impaired glucagon response to hypoglycaemia. One possibility is that the prolonged hyperglycaemia had reduced the number of glucagon producing cells, which would lower the amount of released glucagon as inferred from studies in the obese hyperglycaemic mouse (Hellman 1965). However, this possibility is unlikely in the present study, since increased rather than reduced pancreatic content of glucagon has been observed in this model of diabetes (Tomita *et al.* 1986). Another reason for reduced glucagon secretion in streptozotocin-diabetic rats is impaired secretion secondary to the reduced plasma adrenaline response to hypoglycaemia (Patel 1983a). This is also, however, an unlikely explanation since the impaired adrenaline response, but not the reduced glucagon response, is restored by insulin treatment (Patel 1983b). Another possibility is reduced function of the parasympathetic nerves, since it has been demonstrated that carbachol reverses the impaired plasma glucagon response to hypoglycaemia at 80–100 days after streptozotocin in rats (Patel 1984b). Furthermore, the glucagon response to electrical vagal nerve activation has been shown to be absent, yet that to arginine stimulation is present at 12 weeks after streptozotocin (Hertelendy *et al.* 1992).

An alternative explanation is that reduced A-cell responsiveness to adrenaline or the cholinergic neurotransmitter contributes to the impaired glucagon responses to hypoglycaemia in diabetes. In this study, we examined the glucagon secretory responses to direct adrenergic receptor stimulation by adrenaline and direct muscarinic stimulation by the cholinergic agonist, carbachol. The study was performed at a time point after streptozotocin (6 weeks) when there was $\sim 45\%$ reduction of the glucagon response to insulin-induced hypoglycaemia. We found that the 20 min infusion of adrenaline did not significantly alter the plasma glucose levels in the fasted rats, which is different from the slight hyperglycaemia observed during this period of time in the fed rats (Woodson & Potter, 1979). In addition, we found that the adrenaline infusion increased plasma glucagon levels in both control and diabetic rats. The glucagon response to adrenaline was significantly exaggerated compared with control rats. This confirms a previous study in diabetic humans, who also exhibited hyperresponsive glucagon secretion during adrenaline infusion (Benson *et al.* 1977). The mechanism behind adrenaline-induced glucagon secretion involves increased formation of cyclic AMP and raised concentration of cytoplasmic calcium (Johansson *et al.* 1989). Whether these signalling pathways are altered in streptozotocin-diabetic rats remains, however, to be studied. In any case, our results suggest that the impaired adrenaline response to hypoglycaemia in diabetes (Patel 1983a) might be compensated by an exaggerated A cell response to the catecholamine. Therefore, we conclude that the reduced A-cell responses to direct adrenergic stimulation by adrenaline is unlikely to be involved in the deterioration of the glucagon response to hypoglycaemia in streptozotocin-diabetic rats.

In contrast, we did find that the glucagon response to carbachol was markedly reduced in the streptozotocin-diabetic rats compared with the controls. This result is consistent with the observation that glucagon secretion in response to vagal nerve stimulation is reduced in streptozotocin-diabetic rats (Hertelendy *et al.* 1992) and suggests that reduced direct cholinergic stimulation of glucagon secretion from the A-cell could contribute to the impaired glucagon response to hypoglycaemia in streptozotocin-diabetic rats. The mechanism behind the reduced A cell responsiveness to carbachol in streptozotocin-diabetic rats remains to be established. It is of interest, however, that a previous *in vitro* study in isolated islets derived from streptozotocin-diabetic guinea-pigs has shown a reduced binding of methylscopolamine

to the islets and reduced acetyl choline-stimulated glucagon secretion (Östenson & Grill 1985).

In conclusion, our present study shows that at 6 weeks after administration of streptozotocin in rats, the plasma glucagon response to hypoglycaemia is reduced by ~ 45% and that this coincides with a reduced glucagon response to stimulation with the cholinergic agonist carbachol. In contrast, the glucagon response to adrenaline is exaggerated. The results suggest that reduced A-cell responsiveness to cholinergic but not to adrenergic stimulation may contribute to the impairment of the glucagon response to insulin-induced hypoglycaemia in streptozotocin-diabetic rats.

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