Mechanism of glucoregulatory responses to stress and their deficiency in diabetes

(glucose metabolism/carbachol/third ventricle)

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ABSTRACT During exercise, increased energy demands are met by increased glucose production that occurs simultaneously with the increased glucose uptake. We had previously observed that, during exercise, metabolic clearance rate of glucose (MCR) increases markedly in normal, but only marginally in poorly controlled diabetic dogs. We wished to determine (i) whether in a more general model of stress matched increases in rate of appearance of glucose and MCR also occur, or if MCR is suppressed, as during catecholamine infusion; and (ii) whether diabetes affects stress-induced changes in rate of glucose appearance and MCR. Therefore, we injected carbachol (27 nmol/50 μl), an analog of acetylcholine, intracerebroventricularly in seven conscious dogs before and after induction of alloxan diabetes. In normal dogs, plasma epinephrine and cortisol increased 4- to 5-fold, whereas nor-epinephrine and glucagon doubled. Plasma insulin, however, remained unchanged. Tracer-determined hepatic glucose production increased rapidly, but transiently, by 2.5-fold. This increment can be fully explained by the observed increments in the counterregulatory hormones. Surprisingly, however, MCR also promptly increased, and therefore, plasma glucose changed only marginally. After induction of diabetes, the animals were given intracerebroventricular carbachol while plasma glucose was maintained at moderate hyperglycemia (9.0 ± 0.4 mM). Increments in counterregulatory hormones were similar to those seen in normal dogs, except for exaggerated noradrenaline release. Peripheral insulin levels were higher in diabetic than in normal dogs; however, MCR was markedly reduced and the lipolytic response to stress increased, indicating insulin resistance. Interestingly, the hyperglycemic response to stress was 6-fold greater in diabetic than normal animals, relating mainly to the failure of MCR to rise. Plasma lactate increased equivalently in diabetic and normal animals despite suppression of MCR in the diabetics, indicating either greater muscle glycogenolysis and/or impairment in glucose oxidation. We conclude that in this stress model MCR increases as in exercise in normal but not in diabetic dogs. We speculate that glucose uptake in stress could be mediated through an insulin-dependent neural mechanism.

The obligatory requirement of the brain for glucose necessitates a precise mechanism for glucose homeostasis. The hormonal and metabolic responses to stress have been examined under a variety of stress conditions such as severe injury (1, 2), major surgery (3, 4), myocardial infarction (4, 5), and emotional stress (6); however, the glucoregulatory mechanisms are not fully understood. In exercise, another form of stress, the peripheral energy requirements necessitate changes in glucose metabolism, whereby the augmented rate of glucose utilization (glucose disappearance; RGl) is precisely matched with an elevated rate of hepatic glucose production (glucose appearance; Rg), thus averting the threat of hypoglycemia (7). There is also a safeguard against hyperglycemia dependent on insulin. With insulin deficiency or resistance the exercise-induced increment in metabolic clearance rate of glucose (MCR) is diminished, whereas, depending on the diabetic state, the increment in Rg is often normal—leading to further deterioration of glycemic control (7, 8). We previously attempted to reproduce a portion of the stress response in dogs by epinephrine infusion (9–11): progressive hyperglycemia resulted because of enhanced glucose production and suppressed glucose clearance. In diabetes the hyperglycemia was further exacerbated with epinephrine infusion (10, 11).

A general model of stress was developed in anesthetized rats (12). The intracerebroventricular (i.c.v.) injection of carbachol, an acetylcholine analog, elicits hormonal responses similar to those seen in clinical stress and in exercise. In studies in the rat, glucose fluxes were not examined and, therefore, the mechanism of the ensuing hyperglycemia could not be determined. In addition, it is well known that anesthesia can affect both hormonal responses and their peripheral effects (13).

The aim of the present study is to ascertain the glucoregulatory responses to this moderate general stress (i.c.v. carbachol) in physiology and diabetes. Specifically, we wished to determine (i) whether the expected increase in glucose production induced by release of counterregulatory hormones would also be accompanied by suppression of MCR, as during epinephrine infusions, or if the increased energy demands would lead to an increase in MCR as seen in exercise; and (ii) whether excessive hyperglycemia occurs in diabetic dogs, and if so, whether the hyperglycemia results from excessive glucose production and/or suppressed glucose clearance. Therefore, we injected a small dose of carbachol into the third ventricle via a chronic indwelling i.c.v. cannula in conscious dogs before and after induction of alloxan diabetes. Non-steady-state tracer methods were used to measure glucose production and utilization. In diabetic dogs, basal glucose was clamped at ~9.0 mM with a subbasal peripheral infusion to mimic the hyperglycemia frequently seen in postabsorptive diabetic patients.

MATERIALS AND METHODS

Experimental Animals. Studies were conducted in seven mongrel dogs, 18–26 kg, that were housed under controlled temperature and light cycle conditions and fed once daily a diet of dry chow (lab canine diet 5006; Purina Mills, St. Louis, Missouri).

Abbreviations: Rg, rate of glucose appearance; RGl, rate of glucose disappearance; MCR, metabolic clearance rate of glucose; i.c.v., intracerebroventricular.

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MO) mixed with canned meat (Friskies, Don Mills, ON Canada). Food intake, body weight, body temperature, and hematocrit were monitored regularly. Because extended maintenance of animals with chronic indwelling catheters requires use of antibiotics (14), either penicillin G (Penlong XL; Rogar/STB, Montreal) or gentamicin (Gentocin; Schering) was administered as needed.

Surgery was done under general anesthesia to implant an i.c.v. guide cannula into the third ventricle (15) and vascular sampling and infusion catheters. Two to three weeks elapsed before the first experiment was performed. The patency of the head cannula was verified by aspirating a small volume of cerebrospinal fluid before each experiment. Experiments were done before and after the induction of alloxan diabetes (65 mg/kg; Sigma). Diabetic dogs were maintained on regular and NPH pork insulin (Eli Lilly) injected s.c. daily during morning feeding to keep glucosuria between 0.1 and 1%. At least 6 days was allowed between experiments. All experiments were performed on 16- to 18-hr fasted, conscious dogs trained to lie quietly.

**Experimental Protocol. Normal dogs.** Experiments were begun with a priming injection (25 μCi per 5 ml; 1 Ci = 37 GBq) and constant infusion (0.25 μCi per min) of D-[3-3H]glucose (New England Nuclear). Glucose was diluted to 2.5 μCi/ml in a solution containing unlabeled D-glucose at 100 mg/deciliter (BDH) as carrier and sodium benzoate at 200 mg/deciliter (Fisher) as preservative. Carbachol (carbamoylcholine; Aldrich) at 27 nmol per 50 μl of water was injected i.c.v. after a 2-hr tracer equilibrium period. Injections were made through a 24-gauge injection cannula connected to a 100-μl microsyringe (Hamilton) via polyethylene tubing (Intramedic PE 50; Clay Adams). The head-cannula retaining screw was loosened to allow the needle to pierce the septum and extend ~1 mm beyond the guide cannula.

**Diabetic dogs.** Experiments were done only when the morning plasma glucose was between 8.9 and 13.4 mM. An insulin infusion was begun to lower the plasma glucose levels, which were clamped between 8.9 and 10.0 mM (moderate hyperglycemia). This level of glycaemia during the carbachol experiments was below the urinary threshold in dogs, and therefore, a correction for glucosuria in diabetic dogs was not necessary. Once the desired level was achieved, the insulin infusion rate was held constant, and the experimental protocol proceeded as outlined above for the normal animals. The infusion was prepared from regular pork insulin (100 units per ml, Iletin II) in 50 ml of normal saline containing 2–3 ml of the dog’s own plasma.

**Control studies.** Five normal dogs also received i.c.v. injection of 100 μl of water to ascertain that any effects of the i.c.v. carbachol were not merely a reflection of the pressure effect of the volume of fluid injected.

**Blood Sampling and Assay Methods.** Blood samples for determination of plasma glucose, glucose specific activity, insulin, cortisol, lactate, and glycerol were collected in tubes containing heparin (50 units per ml of blood) and NaF (1–2 mg per tube). Samples for determination of plasma glucagon and free fatty acid concentration were collected in tubes containing 0.1 ml of aprotinin (Trasylol; FBA Pharmaceuticals, New York) and 0.1 ml of EDTA (24 mg/ml; BDH). For determination of norepinephrine and epinephrine, 1-ml blood samples were collected in polyethylene tubes containing 2.5 mg of glutathione (Boehringer Mannheim) and 10 μl of EGTA (Sigma). All blood was stored at 4°C and centrifuged within 60 min. The resultant plasma was stored at −20°C, except the plasma for catecholamine measurements, which was deproteinated with 2 M HClO4 and stored at −70°C.

Glucose concentration, specific activity, and concentration of metabolites (glycerol, free fatty acid, lactate) and hormones (glucagon, epinephrine, norepinephrine) were determined as described (16).

**Calculations.** Glucose concentration and specific activity data were systematically smoothed using the optimal segments technique (17). The $R_1$ and $R_2$ of glucose were calculated with an equation for non-steady-state turnover (18) that was subsequently validated (19). MCR was calculated by dividing $R_2$ by the prevailing plasma glucose concentration. MCR represents an estimate of glucose utilization, partially corrected for the mass action effect of glucose. The validity and meaning of this measurement have been reviewed (20).

**Statistical Analysis.** Values presented are means ± SEMs. Statistical analysis to assess responses to i.c.v. carbachol and differences in response in the diabetic as opposed to the normal state was performed by using a two-way analysis of variance for unbalanced data (Statistical Analysis System; SAS Institute, Carey, NC), run on a microcomputer (IBM P/S 2). Significance was assumed at $P < 0.05$.

**RESULTS**

**Normal Dogs.** As seen in Fig. 1, the i.c.v. injection of carbachol in normal dogs resulted in a marked rapid, but transient, increase in $R_1$ ($P < 0.0001$) from a basal value of 15.8 ± 2.1 to a peak value of 35.8 ± 6.4 μmol/min per kg at

**Fig. 1.** The effect of carbachol (27 nmol per 50 μl of water) injected into the third ventricle ($t = 0$ min) of normal (●) and moderately controlled diabetic (○) dogs ($n = 7$) (mean ± SEM).
10 min. $R_4$ then fell to 60% of the initial response and gradually approached basal levels over 2 hr. There was a concomitant increase in $R_d$ ($P < 0.0001$), which rose from 14.8 ± 2.3 to 26.6 ± 6.5 mg per min per kg. Because $R_d$ and $R_4$ both rose, there was only a marginal increase in plasma glucose concentration (5.9 ± 0.2 to 6.7 ± 0.3 mM; $P < 0.0001$), and consequently, MCR mirrored $R_d$. Concurrently with $R_d$ ($P < 0.0001$), plasma lactate increased 3-fold from 1.16 ± 0.15 to 3.55 ± 1.07 mM. Plasma glycerol concentration doubled from 95 ± 15 to 187 ± 46 μM ($P < 0.0001$), and free fatty acids levels also rose (815 ± 138 to 1321 ± 265 microequivalents per liter; $P < 0.01$) (Fig. 2).

Fig. 3 shows a rapid 2-fold increase over basal in plasma norepinephrine concentration (1.06 ± 0.33 to 1.88 ± 0.37 nM; $P < 0.0001$) and a 3- to 4-fold delayed increase in epinephrine (0.66 ± 0.18 to 2.44 ± 0.64 nM; $P < 0.0001$). There was a marked delayed increase in plasma cortisol (34 ± 13 to 188 ± 42 nM; $P < 0.0001$) and a modest increase in plasma glucagon (151 ± 21 to 247 ± 65 ng/liter; $P < 0.05$). In contrast, insulin remained unchanged from a basal level of 85 ± 13 pM.

Diabetic Dogs. Results seen after the induction of alloxan diabetes are presented together with those in the nondiabetic state. The desired basal plasma glucose concentration of 9.0 ± 0.4 mM (moderate hyperglycemia) was achieved with an average peripheral insulin infusion rate of 137 ± 23 micro-units per min/kg, which caused moderate peripheral hyperinsulinemia (117 ± 22 pM). This infusion rate is ≈80% that required to achieve normoglycemia in depancreatized dogs (11). Basal MCR in these moderately hyperglycemic dogs was markedly suppressed from that in normal dogs (1.58 ± 0.18 ml per min/kg compared with 2.59 ± 0.33; $P < 0.0001$), indicating insulin resistance.

The i.c.v. administration of carbachol to diabetic dogs caused a marked and sustained hyperglycemic response that was six times greater than that in normal dogs. Fig. 1 indicates the main reason for exaggerated hyperglycemia was that, unlike the situation in normal animals, the increase in $R_d$ was not matched by a comparable increase in $R_4$, and MCR remained unchanged from the already greatly diminished basal rate. The more prolonged peak in $R_4$ may also have contributed to hyperglycemia. In diabetic dogs $R_d$ rose from 14.1 ± 1.0 to 21.4 ± 2.3 ($P < 0.0001$). This increment in $R_d$ of the diabetic group was significantly less than that of the normal group ($P < 0.0001$). Fig. 2 shows that the increase in lactate (1.34 ± 0.28 to 3.97 ± 0.83 mM; $P < 0.0001$) was similar to that in normal dogs, despite a much smaller and delayed increase in $R_d$. There was a rapid and marked 4-fold increase in plasma glycerol from 113 ± 25 to 444 ± 81 μM ($P < 0.0001$) that was much larger than in normal dogs. There was also an increase in free fatty acids from 1124 ± 308 to 1718 ± 284 microequivalents per liter ($P < 0.0001$).

The magnitude and dynamics of the responses of plasma epinephrine, cortisol, glucagon, and insulin in the diabetics (Fig. 3) were remarkably similar to that of the normal dogs. However, the norepinephrine response was almost double in diabetic (1.06 ± 0.21 to 2.67 ± 0.20; $P < 0.0001$).
Control Studies. A control study in which water was injected i.c.v. was done in five normal dogs; there was no significant change in any of the hormonal or metabolic parameters measured (data not shown).

DISCUSSION

This study was designed to investigate the hormonal and gluco- regulatory responses to carbachol injection into the brain third ventricle, which provides a model of moderate stress, and to assess alterations in these responses in diabetic dogs. Stress can be classified as a state in which physiological homeostasis is perturbed. However, there are many different types of stress, each characterized by different hormonal and metabolic responses. The activation of the sympathetic nervous system and the anterior pituitary–adrenocortical axis is common to all types of stress. It has been proposed that stimulation of corticotropin-releasing factor can activate the adrenergic corticotropin (ACTH)–cortisol system and the auto- nomic nervous system, concurrently (21). In different types of stress, the ratio of the various counterregulatory hormones released varies. Elevated epinephrine and norepinephrine levels are seen during major surgery (3, 4), trauma (1, 2), severe infections (1, 2), myocardial infarctions (4, 5), ketoads (1), and also emotional stress (6). However, under certain stress conditions, the adrenomedullary activity can be stimulated more than the sympathetic outflow (22, 23). We have observed that in insulin-induced hypoglycemia, plasma epinephrine increased 40-fold, whereas norepinephrine and cortisol increased only 4-fold (24).

Exercise can also be considered as a type of stress. During moderate exercise in normoglycemic dogs, there are 2- to 3-fold proportional increases in epinephrine, norepinephrine, and cortisol (25). However, even with very mild hypoglycemia, increments of epinephrine and cortisol, but not norepinephrine, are generally exaggerated (25). In alloxan diabetic dogs (partial insulin deficiency), responses of all counterregulatory hormones (norepinephrine, epinephrine, cortisol, and glucagon) were increased (26, 27).

Normal Dogs. The i.c.v. administration of a small dose of carbachol seems to provide a reasonable model for a general- ized, moderate stress because most of the generally ac- cepted stress hormones were released. Temporally the peak in plasma norepinephrine preceded that of epinephrine, cor- tisol, and glucagon. This relationship is not unexpected because norepinephrine release reflects increased sympa- thetic neural activity (28), which, in turn, stimulates the release of the other hormones from the adrenal medulla and cortex and pancreatic alpha cells, respectively. Interestingly, no change occurred in plasma insulin, which could reflect a balance between the opposing effects on the pancreatic beta cell of (i) the alpha- and beta-adrenergic effects of the catecholamines or (ii) the adrenergic or cholinergic effects (29). The possibility of parasympathetic involvement, in addition to sympathetic involvement, arises from the fact that pupil dilation and vasodilatation were not observed with car- bachol administration. Concurrently, with the increase in counterregulatory hormones there was a 2-fold increase in glucose production that then gradually declined toward basal. We suggest that this increase in glucose production resulted from the 4-fold increase of epinephrine that was accompanied by a 2-fold increase in glucagon. This result compares well to our previous experiments in depancreatized dogs that were maintained normoglycemic with a constant insulin infusion (11). During epinephrine infusion in these dogs, the incre- ments in glucagon and in glucose production were similar to the responses occurring after i.c.v. carbachol. It is unlikely that increments in cortisol played a role during the 2-hr experimental period because corticol effects are usually not manifested for 3–4 hr (30). It was surprising, however, that the increase in glucose production was accompanied by a matched increment of glucose uptake, particularly because there was no change in plasma insulin, the only hormone known to increase glucose uptake. Therefore, the concomi- tant increase in $$R_g$$ and $$R_d$$ resulted in only a minimal, but significant, increase in plasma glucose concentration. Under similar conditions mimicked by epinephrine infusion, a 2-fold rise in plasma insulin elicited only a minimal increase in $$R_g$$ (9). Indeed, in early studies (31) we have demonstrated that a 2-fold increase in MCR requires a 5- to 6-fold increase in peripheral insulin concentration. Our insulin assay can detect much smaller changes than that and therefore, the increase in MCR cannot be related to the change in peripheral insulin concentration. It is known that peripheral insulin does not accurately reflect the changes in portal insulin, but for peripheral MCR it is peripheral and not portal insulin that is relevant. With epinephrine infusion in human (32, 33) and dog (11), MCR decreased, while in the present study, i.c.v. carbachol injection increased MCR. As a consequence, plasma glucose increased markedly during epinephrine infu- sion but only marginally during carbachol injection. This increase in MCR after carbachol is similar to that seen during exercise in normal dogs, where insulin exerts only a permiss- ive effect (25). The rapid increment in MCR after carbachol implies the possibility of a neural mechanism that could increase glucose uptake independently of any increment in plasma insulin.

Carbachol injection not only mobilized glucose, but it also increased lipolysis, as evidenced by increase of plasma glycerol and free fatty acids.

Diabetic Dogs. The metabolic effects of i.c.v. carbachol were greatly exaggerated in diabetic dogs. The increment in plasma glucose was six times, and that of glucagon was two and one-half times larger than in normal dogs. However, of the hormones measured, only the response of norepinephrine was exaggerated in diabetes. Because the diabetic dogs were given a constant insulin infusion, that insulin concentration did not change is not surprising. The initial rise of plasma glucose could have been, in part, from a more prolonged peak response of hepatic glucose production. However, the main reason for hyperglycemia is that the increment in $$R_d$$ was delayed and much smaller than in normal dogs, and MCR remained suppressed. Therefore, the glucoregulatory effects of i.c.v. carbachol were comparable to those with epinephrine infusion in diabetic, hyperglycemic dogs (11). $$R_d$$ probably rose due only to the mass action of glucose because MCR remained unchanged. Plasma lactate rose similarly in both diabetic and normal dogs. Because in diabetic dogs the $$R_d$$ rise was much smaller than in normal dogs, more lactate could have been produced from greater adrenergic stimulation of muscle glycogenolysis and/or glucose oxidation could be impaired in diabetic animals. The latter is consistent with a defect in glucose oxidation apparently from decreased pyruvate dehydrogenase activity, as seen in longstanding diabetes (34, 35). The former observed was not car- bachol-induced moderate hyperglycemia in diabetic dogs led to peripheral insulin levels higher than in normal dogs. This is not unexpected because insulin was infused peripherally and not intraportally. However, despite such peripheral insulin levels, MCR was markedly reduced, and stress-induced lipolysis was enhanced, indicating insulin resistance. Because of in- sulin resistance, carbachol did not increase glucose clearance in diabetic dogs, as it did in normal dogs. Thus, normal increases in glucose uptake during both stress and exercise appear to be insulin dependent. Our experimental design does not permit us to delineate the site of increased glucose uptake in normal dogs. One can surmise that, in addition to muscle, there would also be some uptake in adipose tissue as illus- trated in Fig. 2 because during carbachol stimulation, in
addition to lipolysis, some reesterification for free fatty acids is indicated as well.

In conclusion, in normal dogs, moderate stress induced by i.c.v. carbachol resulted in the release of all counterregulatory hormones, but the increases in epinephrine and cortisol were most prominent. Increments in glucose production and clearance were matched, and therefore, plasma glucose increased only marginally. The increase in glucose production reflects the changes in the counterregulatory hormones. We speculate, however, that the increment of glucose clearance could reflect a hitherto unknown neural mechanism. It is interesting that glucose regulation during stress and exercise is remarkably similar, which could reflect increased fuel demands occurring under both sets of conditions. In moderately controlled diabetes there was insulin resistance, evidenced by suppressed metabolic glucose clearance and by excessive, stress-induced lipolysis. After i.c.v. carbachol, only the release of norepinephrine was exaggerated. However, the glycemic response is augmented 6-fold, as compared to normal dogs. This result occurred mainly because glucose clearance did not increase. We suggest that, as in exercise, regulation of glucose uptake during stress requires a permissive effect of insulin.

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