Effects of Electric Stress on Glucose Metabolism, Glucose-Stimulated Cyclic Adenosine 3',5'-Monophosphate Accumulation and $^{45}\text{Ca}^{++}$ Efflux in Isolated Pancreatic Islets from Rats Fed with a High Fat Diet

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Synopsis

The effects of the electric stress on glucose oxidation, cyclic adenosine 3', 5'-monophosphate (AMP) accumulation and $^{45}\text{Ca}^{++}$ efflux in response to glucose were studied in pancreatic islets isolated from rats fed on a control (C) or a high fat diet (F) for 12 weeks.

The half of rats on each diet were subjected to electrical shocks in the random time schedule for 1 hr per day for the last 3 weeks of the feeding period (group C-S and F-S). The remaining rats were not given any shocks (group C-NS and F-NS). The rats in F-S group had the high levels of plasma epinephrine, dopamine and blood glucose. The basal content of cyclic AMP after 20 min of incubation with 2.8 mM glucose was decreased in islets from F-S group without affecting insulin release. After 20 min of incubation with 25 mM glucose, the cyclic AMP content in islets from F-S group, which was identical with that in F-NS group, was only 50% of that in C-S group. Insulin release in response to high glucose was significantly inhibited in islets from F-S group. In spite of a remarkable increase of cyclic AMP content in islets from C-S group, insulin release did not differ from that in C-NS group. Glucose (16.7 mM)-stimulated $^{45}\text{Ca}^{++}$ efflux from the perfused islets was greatly inhibited by the high fat diet rather than by stress. The rate of glucose oxidation with 16.7 mM glucose was decreased in islets from F-S group.

It is suggested that the decreased insulin release in response to glucose provoked by the combined effects of the feeding of a high fat diet and electric stress may be mediated by changes of the adenylate cyclase-cyclic AMP system on the plasma membrane of the B-cell or be related to changes in glucose metabolism in islets.

We have previously demonstrated (Yamaguchi *et al.*, 1978) that the combined effects of the long-term feeding of a high fat diet and the stresses induced by electrical shocks result in the impairment of glucose-induced insulin release from the isolated perfused pancreas in the rats, and suggested that the combined effects may be associated with changes in the B-cell function, such as glucose metabolism (Hedekov and Capi-to, 1974; Sener *et al.*, 1976), cyclic AMP levels (Charles *et al.*, 1973; Grill and

Cerasi, 1974; Zewalich *et al.*, 1975) and the translocation of calcium (Brisson and Malaisse, 1973; Sehlin, 1976) in the islets, which are important in regulating insulin release. Furthermore, it is likely that such metabolic changes in the islets are also affected by catecholamines released during the period of stress (Wright and Malaisse, 1968).

It has been suggested that the altered insulin secretory response to glucose in pre-diabetes in man (Cerasi and Luft, 1969), in rat islets after starvation (Howell *et al.*, 1973) and in islets of diabetic Chinese

hamsters (Rabinovitch *et al.*, 1976) may be related to a defect in adenylate cyclase system in the B cell, which may play a role in maintaining optimal cyclic AMP concentration for an appropriate secretory response. Selawry *et al.* (1973) have reported that a drop in the cyclic AMP level in the islets after starvation can be attributed to B-cell response to the increased catecholamine release which takes place during starvation. The inhibitory effects of epinephrine on insulin secretion in rats seem to be mediated by activating α -adrenergic receptor in the islets (Turtle *et al.*, 1967b); α -adrenergic agonist, epinephrine, which inhibits adenylate cyclase activity (Howell and Montague, 1973) lowered cyclic AMP in isolated islets (Turtle and Kipnis, 1976a; Kuo *et al.*, 1974). In addition, the inhibitory effects of epinephrine on insulin secretion appear to interfere with a partial inhibition of glucose-induced Ca uptake (Wollheim *et al.*, 1977). It has been postulated that the cyclic nucleotides might be required for calcium translocation in the B-cell (Brisson *et al.*, 1972). Sener *et al.* (1975) indicated that glycolysis exerts a tight control on the transport of calcium across the B-cell membrane.

In the present study, we have investigated the possibility that the insulin secretory response to glucose might be modulated by changes of cyclic AMP formation in isolated pancreatic islets and the alternation of Ca transport across the B-cell membrane, and be related to changes in glucose oxidation, and that these metabolic changes in islets might be caused by catecholamines.

Materials and Methods

Animals

Male Wistar rats weighing approximately 110g were fed *ad libitum* for 12 weeks either on a control diet (C) containing 50% starch (57% of calories as carbohydrate, 27% as protein and 14% as fat) or on a high fat diet (F) containing 40% butter (19%

of calories as carbohydrate, 17% as protein and 65% as fat). The half of rats on each diet was subjected to stress in the form of electrical shocks in the random time schedule, one session for 1 hr per day, for the last 3 weeks of the feeding period (group C-S and F-S). The remaining rats were not given any shocks and housed individually (group C-NS and F-NS). The apparatus and procedure for the electrical shocks used in the present study have been described in detail elsewhere (Yamaguchi *et al.*, 1978).

Radioenzymatic assay for plasma catecholamines

Collection of plasma samples: Immediately after the last stress session, rats were killed by decapitation and blood samples were collected in tubes with the solution (pH 6.0–7.4), which contained 90 mg/ml of EGTA and 60 mg/ml of glutathione; 20 μ l of this solution was added to each ml of blood collected. After collection, the plasma was separated by centrifugation at 4°C and stored at –20°C.

Assay procedure: Just before assay the plasma was diluted 1:1 with distilled water. Plasma catecholamines were measured by a method with the radioenzymatic assay kit (The Upjohn Company, U.S.A.). The assay is based on the 3-*o*-methylation of norepinephrine, epinephrine and dopamine by the enzyme catechol-*o*-methyl-transferase (COMT) in the presence of S-adenosyl-L-methionine-[³H-methyl] (³H-SAM). The resulting substances were isolated by thin layer chromatography (TLC plates Silica gel 60-F 254, 0.25-mm thickness, Merck). The radioactivity attributable to each catecholamine was determined by a liquid scintillation spectrometer (Packard, model 3385).

Isolation of pancreatic islets of Langerhans

Pancreatic islets of Langerhans were isolated from rats either fed for 24 hrs or starved overnight after the end of the last stress session, according to the method of Lacy and Kostianovsky (1967). Krebs-Henseleit bicarbonate (KHB) buffer equilibrated with 95% O₂:5% CO₂, and supplemented with 2.8 mM glucose and bovine serum albumin (BSA, 10–16 mg/ml) was used in collagenase digestion of the pancreas.

Insulin release and cyclic AMP content in isolated islets

Pancreatic islets were obtained from rats fed for 24 hrs after the end of the last stress session. After a preincubation period of 30 min at 37°C in the basal medium which consisted of a KHB buffer (pH 7.4) equilibrated with 95% O₂:5% CO₂ containing 2.8 mM glucose and 0.5% BSA, groups of 50 islets were incubated for further 20 min at 37°C in 500 μ l of fresh medium containing 2.8 or 25 mM glucose and 0.5% BSA. At the end of the incubation period the islets were removed from the incubation tubes,

homogenized in 500 μ l of ice-cold 6% trichloroacetic acid (TCA) by the sonication for 40 sec. The homogenate was centrifuged at 3000 rpm at 4°C for 15 min. The supernatant was washed twice with 5 volumes of ethyl ether, and the acid-free solution was used for radioimmunoassay of cyclic AMP (YAMASA Cyclic AMP Assay Kit; YAMASA SHOYU K. K.). Insulin released into the incubation medium was measured by the double antibody radioimmunoassay (INSULIN RIAKIT; Dinabot RI Labs. Ltd.) using rat insulin as a standard.

⁴⁵Ca⁺⁺ efflux from the perfused islets

Pancreatic islets were obtained from rats fed for 24 hrs after the end of the last stress session. The media used for incubation, washing and perfusion were KHB buffer (pH 7.4) equilibrated with 95% O₂:5% CO₂, containing 0.5% BSA. Groups of 100 islets were loaded with ⁴⁵Ca (72.3 mCi/ml; ⁴⁵CaCl₂; The Radiochemical Centre, New England Nuclear, Boston, Mass) in the presence of 2.8 mM glucose during 60 min of incubation at 37°C. After loading, the islets were washed 5–6 times with the non-radioactive incubation medium, and then placed in a small perfusion chamber (vol. 500 μ l) at 37°C. From zero to 32 min the islets were perfused with the basal medium in the absence of glucose. At the 32nd min the glucose concentration was raised to 16.7 mM and the stimulation was continued to the 50 min. The flow rate was 0.5 ml/min. The effluent was collected for every one minute. 300 μ l of samples were collected in counting vials and added with 7 ml of scintillation fluid (PCS; The Radiochemical Centre, Amersham, England) for the assay of ⁴⁵Ca by a liquid scintillation spectrometer (Packard, model 3385). The ⁴⁵Ca efflux (cpm/min) was expressed in per cent of the mean values between the 30th and 32nd.

Glucose oxidation in isolated islets

Pancreatic islets were obtained from rats starved overnight after the end of the last stress session. For the measurement of glucose metabolism, 10 islets were collected in the microtubes with 100 μ l of KHB buffer (pH 7.4) equilibrated with 95% O₂:5% CO₂, containing D-[U-¹⁴C]glucose (specific activity 316 mCi/mmol; The Radiochemical Centre, Amersham, England), 2 or 16.7 mM glucose and 3% BSA. The microtubes were placed into counting vials and stoppered. The vials were incubated with shaking at 37°C for 2 hrs. After the incubation, the reaction was terminated by injecting 50 μ l of 1 N H₂SO₄ into the microtubes containing the islets, and after injecting 500 μ l of Hyamine into the outer vial the ¹⁴CO₂ was absorbed for 1 hr at room temperature. Blanks consisted of the incubation medium without islets were carried out through the whole procedure and the blank radioactivity was subtracted from the

experimental value, as described by Ashcroft *et al.* (1970). The radioactivity in Hyamine was measured by a liquid scintillation spectrometer (Packard, model 3385).

Results

Body weight

The rats in F-NS group (mean $\pm$ SE; 548 $\pm$ 6 g, n=13) gained a significant amount of weight than in C-NS group (514 $\pm$ 6 g, n=12) ($p < 0.001$). The mean body weight in F-S group (497 $\pm$ 6 g, n=11) was significantly heavier than that in C-S group (468 $\pm$ 8 g, n=11) ($p < 0.001$), although the rats on both diets did not gained weight through the period of stress. The inhibitory effect of stress on weight change was not affected by the high fat diet.

Plasma catecholamine levels

The sensitivity of the assay has been shown in the range of 3–5 pg of epinephrine and norepinephrine and 20 pg of dopamine, per 50 μ l of sample. The linearity of the assay was maintained to 3,000 pg, as can be seen in Fig. 1.

The effects of stress on the levels of plasma epinephrine (E), norepinephrine (NE) and dopamine (DA) in rats fed on each diet are shown in Fig. 2. Under non-stress conditions, plasma levels of each catecholamine were not altered by the high fat diet. Immediately after the end of the last stress session, the levels of plasma E in the rats on both diets was significantly elevated than in NS groups. The increased level of plasma E in F-S group was identical with the level in C-S group. The level of plasma NE was not significantly elevated by stress. A remarkable increase in plasma DA after stress was observed in the rats fed on a high fat diet ($p < 0.025$) but not on a control diet. At the same time, the levels of plasma glucose were 124 $\pm$ 6 mg/100 ml (mean $\pm$ SE; n=6) in C-NS group, 129 $\pm$ 2 mg/100 ml (n=5) in F-NS

Fig. 1. Sensitivity and linearity of the assay with catecholamines. The catecholamine standard solution, which contains 100 $\mu\text{g}/\text{ml}$ of each catecholamine (*l*-norepinephrine, *l*-epinephrine and dopamine) in acidic glutathione solution, were diluted with distilled water. Blank consists of 50 μl of distilled water. The radioactivity of ^3H -labeled-*o*-methylated substrate of each catecholamine (^3H -normetanephrine, ^3H -metanephrine and ^3H -3-metoxytyramine, respectively) was measured. Each point represents the mean value of three to four determinations subtracted blank. Solid lines represent linear regression line for epinephrine and norepinephrine, and broken line for dopamine. Correlation coefficients are 0.999 for epinephrine, 0.996 for norepinephrine and 0.999 for dopamine.

group, $134 \pm 3 \text{ mg}/100 \text{ ml}$ ($n=6$) in C-S group and $140 \pm 4 \text{ mg}/100 \text{ ml}$ ($n=6$) in F-S group.

Insulin release and cyclic AMP content in isolated islets

The intracellular concentration of cyclic AMP accumulated in islets and insulin released into the medium during 20 min of incubation in the presence of 2.8 or 25 mM glucose are given in Table 1. The basal content of cyclic AMP and insulin release in islets at 2.8 mM glucose were not affected by the high fat diet. In C-S group, stress markedly increased the basal insulin release, but there was no detectable increase of cyclic AMP content in islets. The basal

Fig. 2. Effects of stress on the levels of plasma catecholamines in rats fed on control (A) and high fat diet (B). The stressed rats were sacrificed by decapitation immediately after the end of stress session. The non-stressed rats were sacrificed at the same time. Each bar and vertical line represent the mean $\pm$ SE. The number of observations is indicated in parentheses.

content of cyclic AMP in islets from F-S group was reduced to 49% of that in F-NS group ($p<0.02$), but the inhibitory effect on insulin release was not observed. When the glucose concentration of the medium was raised to 25 mM, the cyclic AMP content in islets were twofold increased over the levels in islets at 2.8 mM glucose in both C-NS group and F-NS group, corresponding to the increase of insulin release. In C-S group, the cyclic AMP content in islets at 25 mM glucose was stimulated five fold over the level at 2.8 mM glucose which was higher than that in C-NS group ($p<0.005$). However, insulin release in response to glucose in C-S group was similar to that in C-NS group. In F-S group, the cyclic AMP content in islets stimulated with 25 mM glucose did not differ from that in F-NS group but was only 50% of that in C-S group ($p<0.005$). Insulin release in response to high glucose (25 mM) in F-S group was decreased by 63% of that in F-NS group ($p<0.05$).

Table 1. Glucose-stimulated insulin release and elevation of cyclic AMP in isolated rat islets.

	Insulin release (ng/50 islets/20 min)				cAMP in islets (pmol/50 islets)			
	2.8 mM		25 mM		2.8 mM		25 mM	
Glucose								
Non-stress								
Control	11.6±1.3	(3)	34.9±4.9	(8)	0.40±0.10	(3)	0.75±0.08	(8)
High fat	14.3±4.1	(2)	38.6±8.1	(6)	0.45±0.07	(2)	0.90±0.06	(6)
Stress								
Control	23.3±1.1****	(3)	35.5±3.8	(9)	0.39±0.09	(3)	1.53±0.24****	(4)
High fat	16.2±1.7 ^{ooo}	(4)	24.4±2.2* ^{oo}	(9)	0.22±0.04**	(3)	0.81±0.10 ^{ooo}	(9)

Batches of 50 islets were used. After 30 min of preincubation with 2.8 mM glucose, insulin released into the incubation medium and cyclic AMP levels in islets during 20 min of incubation with 2.8 mM or 25 mM glucose were measured. Values are means±SE. The number of observations is indicated in parentheses.

* Significantly different from non-stress group in each diet group, $p<0.05$,

** $p<0.02$, **** $p<0.005$.

^{oo} Significantly different from control in stress group, $p<0.02$, ^{ooo} $p<0.01$, ^{oooo} $p<0.005$.

Glucose-stimulated $^{45}\text{Ca}^{++}$ efflux from the perfused islets

As shown in Fig. 3, when 16.7 mM glucose was added to a perfusate in the absence of glucose at the 32nd min, a bi-phasic pattern of $^{45}\text{Ca}^{++}$ efflux was observed. In C-S group (upper left panel), after the glucose stimulation an initial retention of $^{45}\text{Ca}^{++}$ outflow was observed within 2 min and the peak values in $^{45}\text{Ca}^{++}$ efflux were observed at the 6th min after the glucose stimulation. The efflux of $^{45}\text{Ca}^{++}$ then progressively declined and was decreased to about 35% of the peak values at the 50th min. In C-S group (upper right panel), although the peak values of $^{45}\text{Ca}^{++}$ efflux ($143\pm7\%$, $n=4$) at the 5th min after the glucose stimulation did not significantly differ from that in C-NS group ($169\pm22\%$, $n=5$). An inhibitory effect of glucose on $^{45}\text{Ca}^{++}$ efflux was clearly observed for a longer period in C-S group than in C-NS group.

A high fat diet reduced glucose-stimulated $^{45}\text{Ca}^{++}$ efflux from the perfused islets (lower panel). The peak values of $^{45}\text{Ca}^{++}$ efflux observed at the 6th min after the glucose stimulation in F-NS and F-S group were significantly inhibited by 73% of that in C-NS group; the peak values of $^{45}\text{Ca}^{++}$

efflux were $123\pm8\%$ ($n=7$) in F-NS group and $123\pm7\%$ ($n=8$) in F-S group. In F-S group (lower right panel), however, the remarkable inhibitory effect of glucose on $^{45}\text{Ca}^{++}$ efflux was shown to be similar to that in C-S group. Insulin release from the perfused islets in response to glucose in F-S group was dramatically diminished (results not shown).

Glucose oxidation in isolated islets

As shown in Fig. 4, the rate of glucose oxidation in islets from fasted rats in the presence of non-stimulatory glucose concentration (2 mM) was unaffected by both the high fat diet and stress. When the glucose concentration of the medium was raised to 16.7 mM, the rate of glucose oxidation was increased and there was no significant difference between the rates in C-NS group and F-NS group. The rate of glucose oxidation at high glucose (16.7 mM) in C-S group was also similar to that in C-NS group. However, the rate of glucose oxidation at high glucose in F-S group was significantly lower than that in C-S group ($p<0.05$).

Fig. 3. The efflux of $^{45}\text{Ca}^{++}$ from the perfused pancreatic islets. Groups of 100 islets were loaded with $^{45}\text{Ca}^{++}$ for 60 min in medium containing 2.8 mM glucose. After loading and washing, the islets were perfused with the basal medium in the absence of glucose. At the end of the 32nd min, the perfusate was switched to medium containing 16.7 mM glucose and was derived to the 50th min. Results are expressed in per cent of the mean value (cpm/min) found between the 30th and 32nd min in each individual experiment. Each point and vertical line represent the mean $\pm$ SE. of values.

Fig. 4. Oxidation of $[\text{U-}^{14}\text{C}]$ glucose in pancreatic islets from rats fasted overnight. Batches of 10 islets were incubated for 2 hrs at 37°C in medium containing 2 mM or 16.7 mM D- $[\text{U-}^{14}\text{C}]$ glucose and 3 mg/ml bovine serum albumin. Each bar and vertical line represent the mean $\pm$ SE. The number of observations is indicated in parentheses. NS, Non-stress group; S, Stress group.

Discussion

The present study demonstrates that the electric stress increases the levels of plasma catecholamines, especially of epinephrine, and in rats fed on a high fat diet characteristically further increases the level of plasma dopamine. It has been shown by Wright and Malaisse (1968) that in rats forced to swim or submitted to repeated electrical shocks, the rate of insulin release is dramatically reduced. It therefore indicates the possibility that endogenous catecholamines released in response to stress exert the inhibitory effect on insulin release in rats fed on a high fat diet, although in rats decapitation itself used in the present study may be caused by a significant increase of all three catecholamines (Bühler *et al.*, 1978). Epinephrine is known to inhibit insulin secretion (Feldman and Lebovitz, 1971), and norepinephrine and dopamine also inhibit insulin secretion through a similar or identical mechanism (Lebovitz and Feldman, 1973). These endogenous monoamines cannot penetrate the cell membrane and act only the cell surface only to interfere with the release of secretory granules on the surface (Lebovitz and Feldman, 1973). Epinephrine inhibits the adenylate cyclase by activating α -adrenergic receptor sites in islets (Porte and Robertson, 1973) and consequently lowers the level of the pancreatic cyclic AMP (Turtle and Kipnis, 1967a).

In the present study, the basal level of cyclic AMP in islets after 20 min of incubation with 2.8 mM glucose was unaffected by the high fat diet under non-stress conditions. However, stress lowered the basal content of cyclic AMP in islets from rats fed on a high fat diet. This lowered cyclic AMP in islets from the stressed rats on a high fat diet does not seem to be ascribed to the direct effect of epinephrine because the plasma level of stress-induced epinephrine did not signifi-

cantly differ from that in the stressed rats on a control diet. It may be caused by the additional effects of the elevated plasma dopamine and/or of a long-term feeding of a high fat diet. On the other hand, it has already been established that the cyclic AMP levels are important in the regulation of glucose-induced insulin release (Zawalich *et al.*, 1975). Our present study indicates that in the absence of the phosphodiesterase inhibitor, the cyclic AMP content in islets during a short time of incubation with high glucose (25 mM) is increased, as reported by Zawalich *et al.* (1975) and Charles *et al.* (1973) and Wolter *et al.* (1977). In rats fed on a control diet, stress produced a marked elevation of cyclic AMP content in islets at high glucose, despite of no significant increase of insulin release. In the stressed rats on a high fat diet, however, the cyclic AMP content in islets at high glucose was enhanced to the level in islets from the non-stressed rats on a high fat diet but was much lower than the level in islets from the stressed rats on a control, and glucose-induced insulin release was inhibited. These results suggest that the decreased insulin release in the stressed rats on a high fat diet may be interfered with by an early defect of the elevation of cyclic AMP to initiate insulin release in response to glucose, and may be mediated by an alteration in the activity of adenylate cyclase in the B-cell.

The precise role of cyclic AMP in the insulin releasing process is not known, but it has been suggested that the insulino-tropic action of cyclic AMP could be due to the translocation of calcium within the B-cell (Brisson *et al.*, 1972). Brisson and Malaisse (1973), in the studies of the efflux of $^{45}\text{Ca}^{++}$ from isolated islets in a perfusion system showed that cyclic AMP and agents which raised cyclic AMP levels could increase the efflux of $^{45}\text{Ca}^{++}$ from the B-cell. In the present study, the efflux of

$^{45}\text{Ca}^{++}$ from the perfused islets was significantly inhibited by the high fat diet rather than by stress. It indicates that the high fat diet influences the efflux of calcium from the B-cell membrane. The finding may be reflected by a difference in the cellular composition of the islets and/or an inhibition of the intracellular redistribution of Ca. However, stress greatly induced an early inhibitory effect of glucose on ^{45}Ca efflux from the B-cell both on a control and a high fat diet. Thus it indicates that stress also has an effect on the distribution of Ca within the B-cell and may affect the intracellular calcium translocation. Therefore it suggests that the decreased insulin release in response to glucose in islets from the stressed insulin release in response to glucose in islets from the stressed rats on a high fat diet may be caused by the intracellular distribution of calcium in the B-cell.

On the other hand, Sener *et al.* (1976) have reported that in the process of glucose-induced insulin release, glycolysis controls the rate of Ca transport across the B-cell membrane. Howell *et al.* (1975) and Sehlin (1976) have showed that the uptake of Ca in islets is stimulated by ATP. It has recently been shown that glycolytic intermediates, phosphoenolpyruvate and fructose 1, 6-diphosphate, inhibit ATP-dependent ^{45}Ca uptake by a mitochondrial fraction from rat islets and might influence changes in the cytosolic calcium concentration and insulin release in the B-cell (Sugden and Ashcroft, 1978). Since the decrease of glucose oxidation was observed on islets from the stressed rats on a high fat diet, if an alteration in the concentration of glycolytic intermediates could be expected, the decreased insulin release in response to glucose would also be related to changes in glucose metabolism in the B-cell.

In conclusion, the long-term combined actions of a high fat diet and electric stress appear to modify the pancreatic B-cell

function and modulate the intracellular redistribution of Ca in the B-cell. The decreased insulin release in response to glucose in islets from the stressed rats on a high fat diet seems to interfere with the inability of glucose to enhance the formation of cyclic AMP in the initial phase of release and a reduced glucose metabolism in the B-cell.

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