EDTA and Insulin*

A Study of the Effect of Salts of EDTA upon Insulin Action *in vivo* and *in vitro*.

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Summary. Insulin induced hypoglycaemia was enhanced in rats, by the simultaneous administration of disodium EDTA or calcium disodium EDTA but not by magnesium disodium EDTA. None of these EDTA complexes affected the blood sugar in the absence of insulin. — *In vitro*, both Na and Ca EDTA inhibited the insulin stimulation of glucose uptake by the isolated rat hemidiaphragm, but had no effect upon basal glucose uptake. In contrast, Mg EDTA increased basal glucose uptake with little or no effect upon insulin stimulation. On the isolated epididymal fat pad, both Na and Ca EDTA either did not affect, or slightly reduced, the insulin stimulation of glucose uptake. They sometimes increased the basal glucose uptake. Mg EDTA consistently increased the basal glucose uptake. — It is suggested that the *in vivo* effect of Ca and Na EDTA is upon the homeostatic response to hypoglycaemia rather than upon the action of insulin.

EDTA et insuline: étude de l'effet de sels d'EDTA sur l'action de l'insuline, in vivo et in vitro.

Résumé. L'hypoglycémie provoquée par l'insuline était augmentée chez les rats par l'administration simultanée d'EDTA disodique ou d'EDTA calco-disodique, mais non par l'EDTA magnésio-disodique. Aucun de ces complexes EDTA ne modifiait le glucose sanguin en l'absence d'insuline. — *In vitro*, l'EDTA de Na et l'EDTA de Ca inhibaient tous les deux la stimulation due à l'insuline, de la captation de glucose par l'hémidiaphragme isolé du rat, mais n'avaient pas d'effet sur la captation basale de glucose. — Par contre, l'EDTA de Mg augmentait la captation basale de glucose avec peu ou pas d'effet sur la stimulation due à l'insuline. Sur le tissu adipeux épидидymaire, l'EDTA de Na et l'EDTA de Ca ou bien ne modifiaient pas, ou bien réduisaient légèrement la stimu-

lation par l'insuline de la captation du glucose. Ils augmentaient parfois la captation basale de glucose. L'EDTA de Mg augmentait nettement la captation basale de glucose. — On suggère que l'effet *in vivo* de l'EDTA de Ca et de Na s'exerce sur la réponse homéostatique à l'hypoglycémie plutôt que sur l'action de l'insuline.

EDTA und Insulin: Untersuchungen des Effektes von EDTA-Salzen auf die Insulinwirkung in vivo und in vitro.

Zusammenfassung. Bei Ratten wurde die insulininduzierte Hypoglykämie durch gleichzeitige Gabe von Dinatrium-EDTA oder Calcium-Dinatrium-EDTA, nicht jedoch von Magnesium-Dinatrium-EDTA, verstärkt. Ohne Insulin beeinflusste keine dieser Verbindungen den Blutzuckerspiegel. — *In vitro* hemmten sowohl Na- als auch Ca-EDTA die insulinstimulierte Glucoseaufnahme des isolierten Rattendaphragma, doch hatten diese Verbindungen keinen Einfluß auf die basale Glucoseaufnahme. Im Gegensatz dazu führte Mg-EDTA zu einer Steigerung der basalen Glucoseaufnahme mit wenig oder fehlender Wirkung auf die insulinstimulierte Glucoseaufnahme. Am isolierten epididymalen Fettgewebe zeigten Na- und Ca-EDTA entweder keinen oder einen leicht hemmenden Einfluß auf die insulinstimulierte Glucoseaufnahme. Manchmal kam es zu einer Steigerung der basalen Glucoseaufnahme. Übereinstimmend steigerte Mg-EDTA die basale Glucoseaufnahme. — Es wird angenommen, daß der *in vivo*-Effekt des Ca- und Na-EDTA eher durch Wirkung auf den homöostatischen Regulationsmechanismus bei der Hypoglykämie als durch Beeinflussung der Insulinwirkung zustande kommt.

Key-words: EDTA, Insulin, Hypoglycaemia, Adipose Tissue, Muscle, Chelating agents.

There is an increased urinary excretion of zinc in some diabetics and it has been suggested that concurrent urinary excretion of chelating metabolites might be responsible for this (RIHAN, 1966). There have also been reports that the administration of chelating agents enhanced the fall in blood sugar induced by insulin in insulin-dependent diabetics (BUTTERFIELD, 1955; MELTZER et al., 1961; LAMAR, 1964). Since there is considerable evidence of a link between insulin and a number of divalent metals, particularly zinc (FISHER and SCOTT, 1935; KADOTA, 1950; MASKE, 1957), it seemed possible that this insulin-enhancing effect of chelating agents might be explained by an effect upon zinc or other divalent ions.

* Based upon a communication to the Medical and Scientific Section of the British Diabetic Association, September, 1965.

This paper presents the results of experiments designed to define the effect of salts of ethylenediamine tetra-acetic acid (EDTA) both *in vivo* and *in vitro*. This chelating agent was investigated because several workers (MELTZER et al., 1961; BHATTACHARYA, 1961, DIXIT and LAZAROW, 1965) have previously reported effects of EDTA on carbohydrate metabolism both *in vitro* and *in vivo*.

Materials and Methods

The rats used in these studies were male Wistar rats, bred in the Animal House at Guy's Hospital Medical School.

For the *in vivo* studies, rats of weight 150 ± 5 g were used.

In each experiment, experimental groups of fed rats were injected intraperitoneally with insulin (0.5

units), an EDTA complex, or insulin + EDTA complex. The control group received an intraperitoneal injection of physiological saline. Blood samples were taken from the warmed tail tip before and at intervals up to 270 minutes after injection, or at death. Glucose estimations were performed with an Auto Analyser, using the method of HOFFMAN (1937).

Rat Hemidiaphragm and epididymal fat incubations were performed in Krebs-Ringer bicarbonate buffer (UMBREIT et al., 1964) containing 2.5 mg/ml glucose and 2.0 mg/ml crystalline bovine albumin. The tissues were prepared as described previously (GARRATT, JARRETT and KEEN, 1966). Hemidiaphragms were incubated in 2.0 ml medium for 90 minutes and epididymal fat pieces, weighing approximately 25 mg in 1.0 ml medium for 180 minutes, at 37°C in 95% O₂ / 5% CO₂ atmosphere in a shaking incubator. Glucose uptake of the tissue was calculated from the fall in glucose concentration of the medium occurring during incubation.

Insulin binding to the hemidiaphragm was measured in the presence and absence of EDTA by the method of GARRATT et al. (1966).

Results

1. In vivo effects. Na, Ca and Mg EDTA, in doses of 20 mg intraperitoneally, had no effect upon the blood sugar measured at 90 minutes and 4½ hours after injection, nor were there any other obvious effects upon the appearance and behaviour of the animals (Table 1a). Doses of 1g were injected in subse-

Table 1. The effect of 20 mg doses of three complexes of EDTA, with and without insulin (0.5 units), upon the blood sugar of fed rats. All injections were made intraperitoneally in a volume of 1 ml

a		Mean deviation of blood sugar from basal (mg 100ml)	
Time after injection (minutes)		90	270
No. of animals	Material injected		
7	Saline (control)	— 7	— 1
18	Insulin 0.5 U	— 55	— 37 ¹
7	Insulin + Ca EDTA	— 75	— 50 ³
8	Insulin + Na EDTA	— 80	— 42 ³
10	Insulin + Mg. EDTA	— 63	— 21 ⁴
8	Na EDTA	— 8	— 1
8	Ca EDTA	— 12	— 1
5	Mg EDTA	— 2	+ 2

1. 5 animals died between 90 and 270 minutes. Blood sugars were determined in two of these.

2. 5 animals died between 90 and 270 minutes.

3. 5 animals died between 90 and 270 minutes.

4. 1 animal died between 90 and 270 minutes.

quent experiments, designed to achieve levels of EDTA comparable with those used *in vitro*. Na and Mg EDTA were not tolerated in this dosage, irritability and te-

tany occurring in all the animals and death in several. Ca EDTA, however, was without these effects. The effect of Na EDTA was also assessed over the 60 minute period following injection. There was no difference between the effect of Na EDTA and that of the control. (Table 1b).

b		Mean deviation of blood sugar from basal (mg 100ml)				
Time after injections (minutes)		60				
No. of animals	Material injected					
4	Saline (control)	+3	+3	+2	+4	
4	Na EDTA	+4	+5	+6	+7	

The blood-sugar lowering effect of intraperitoneal insulin (0.5 units/animal) alone was compared with that of the same dose of insulin injected along with 20 mg EDTA. Both Ca and Na EDTA enhanced the insulin hypoglycaemia; several animals died and the blood sugar values in the survivors were lower at both 90 minutes and 4½ hours after injection in the groups receiving Na and Ca EDTA respectively (Table 1a). By contrast, the same (20 mg) dose of Mg EDTA had no effect upon the response to insulin (Table 1a). Ca EDTA (1g) was also found to enhance the insulin hypoglycaemia.

2. In vitro effects. Hemidiaphragm incubations: Ca and Na EDTA in a range of concentrations from 10⁻⁴ to 10⁻²M had no effect upon the basal glucose uptake of the isolated hemidiaphragm (Fig. 1), but, at the higher concentrations, they completely inhibited the stimulant action of added insulin (1 mU/ml) on glucose uptake (Fig. 1 and 2). By contrast, Mg EDTA

Fig. 1. The effect of Ca EDTA at three concentrations upon basal and insulin-stimulated glucose uptake of rat hemidiaphragm. Each histogram represents the mean ± S.E.M. of three observations

increased basal glucose uptake and only partially inhibited the effect of the added insulin (Fig. 3).

The addition of Mg⁺⁺ at various concentrations failed to restore the response to insulin in the presence of Na or Ca EDTA (Table 2). When hemidiaphragms were incubated in Mg⁺⁺-free Krebs-Ringer bicarbo-

nate buffer, the basal glucose uptake was reduced, but there remained a considerable stimulation of glucose uptake in the presence of insulin (Fig. 3).

Table 2. The effect of Ca EDTA, with and without added Mg^{++} , upon insulin stimulation of glucose uptake by isolated rat hemidiaphragm. Results are the mean $\pm$ S.E.M. of three estimations. Osmolality was maintained, when $Mg Cl_2$ was added, by adjusting the NaCl concentration.

Bicarbonate buffer +	Glucose uptake mg/g
1 Insulin 1 mU/ml.	8.3 ± 1.28
2 Insulin 1 mU/ml. + Ca EDTA ($10^{-2}M$)	3.3 ± 1.31
3 Insulin 1 mU/ml. + Ca EDTA + $Mg Cl_2$ (1 mg./ml)	3.8 ± 1.21
4 Insulin 1 mU/ml. + Ca EDTA + $Mg Cl_2$ (3 mg./ml)	3.7 ± 0.89
5 Insulin 1 mU/ml. + Ca EDTA + $Mg Cl_2$ (9 mg./ml)	4.9 ± 0.56

Fig. 2. The effect of Ca and Na EDTA ($10^{-2}M$) upon the glucose uptake of the rat hemidiaphragm in the presence and absence of insulin. Each histogram represents the mean $\pm$ S.E.M. of three observations

Fig. 3. Glucose uptake of rat hemidiaphragm. The effects of Ca and Mg EDTA ($10^{-2}M$) in normal Krebs-Ringer bicarbonate buffer and the response of the diaphragm in Mg^{++} free buffer. Each histogram represents the mean $\pm$ S.E.M. of three observations

Iodoinsulin binding to diaphragm measured after 2, 5, 10 and 90 minutes of incubation was the same in the presence or absence of Ca and Na EDTA. The EDTA complexes also did not influence the rate of insulin breakdown, estimated from the proportion of trichloroacetic acid — soluble radioactivity appearing during incubation of diaphragm with ^{131}I -labelled insulin.

Epididymal fat incubations: Ca and Na EDTA in concentrations from 10^{-4} to $10^{-2}M$ either had no effect upon basal glucose uptake of epididymal fat or, in

some experiments, caused a slight increase in glucose uptake (Fig. 4). Although, in a few experiments, EDTA diminished the stimulant effect of added insulin upon glucose uptake by the epididymal fat pad, in most experiments it had no effect whatsoever. Complete suppression of the insulin effect was never seen; nor was there any enhancement of the insulin effect, as described by DIXIT and LAZAROW (1965) with Na EDTA.

Mg EDTA ($10^{-2}M$) increased the basal glucose uptake of the fat pad and diminished the incremental increase when insulin was added (Fig. 5).

Fig. 4. The effect of Ca EDTA at three concentrations upon basal and insulin-stimulated glucose uptake of rat epididymal fat. Each histogram represents the mean $\pm$ S.E.M. of three observations

Fig. 5. The effect of Mg EDTA ($10^{-2}M$) upon basal and insulin-stimulated glucose uptake of rat epididymal fat. Each histogram represents the mean $\pm$ S.E.M. of six observations

Discussion

The experiments *in vivo* reported above clearly demonstrate that in the rat both Ca and Na EDTA lower the blood sugar by enhancing the hypoglycaemia produced by exogenous insulin, results which are comparable with those of MELTZER et al. (1961) in man. Mg EDTA, however, does not potentiate the action of insulin, which suggests that the insulin-enhancing effect is not due to the EDTA molecule itself, but to some alteration in ionic balance. What this necessary alteration may be is uncertain. All three forms of EDTA should complex with cations such as Mn^{++} ,

Zn^{++} and Cu^{++} , and it therefore seems improbable that the *in vivo* effect can be attributed simply to chelation of trace metals. The predominant effect of both Na and Mg EDTA is to lower the plasma calcium, yet of these two, only Na EDTA possesses the insulin-enhancing property. NINOMAYA et al. (1966) have recently shown that infusion of Ca or Na EDTA into the pancreatic artery of the dog increases the insulin-like activity in the pancreatic venous blood. Such an effect could not explain our results, however, as there was no effect on the blood sugar of the animals when EDTA was administered alone. In this respect, EDTA differs from another chelating agent, BAL, which PROUT et al. (1958) have shown to be hypoglycaemic, in the rabbit, apparently independently of insulin.

DIXIT and LAZAROW (1965) reported that Na EDTA enhanced the effect of insulin *in vitro* upon the isolated rat epididymal fat pad, results which might have offered an explanation of our *in vivo* findings. We were unable, however, to confirm their results. Na and Ca EDTA either did not affect, or slightly reduced the insulin-stimulated glucose uptake of the isolated fat pad.

The effect of EDTA upon the hemidiaphragm *in vitro* throws no light upon the effects *in vivo*. Both Na and Ca EDTA, which enhanced the action of insulin *in vivo*, inhibited the insulin stimulation of glucose uptake by the hemidiaphragm. This inhibition did not seem to be due to chelation of Mg^{++} ions, as the work of BHATTACHARYA (1961) might suggest. In fact, contrary to the findings of BHATTACHARYA, who used sucrose buffer, we found that Mg^{++} ions were not essential for insulin stimulation of glucose uptake in a bicarbonate buffer. Our experiments with ^{131}I -labelled insulin did not demonstrate any effect of Na or Ca EDTA upon the binding or metabolism of insulin *in vitro*.

Mg EDTA which differed both in the *in vivo* experiments and upon adipose tissue *in vitro*, again behaved differently upon the hemidiaphragm. Unlike Na and Ca EDTA, it increased the basal glucose uptake of the tissue, and partially inhibited the insulin stimulation.

Whatever the explanation of the effects *in vitro* of Ca and Na EDTA may be, these compounds present an interesting parallel with the "synalbumin" insulin antagonist, namely insulin-like activity upon adipose tissue and insulin-inhibitory activity upon the hemidiaphragm (VALLANCE-OWEN, 1964; CAMERON et al., 1966).

An alternative explanation of the *in vivo* effects of Ca and Na EDTA is that they decrease the homeo-

static responses to a fall in blood sugar rather than increase the insulin-induced removal of glucose from the circulation. Further investigation of this aspect would appear to offer most prospect of elucidating the mechanism of action of EDTA.

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