Cadmium-induced stimulation of lipogenesis from glucose in rat adipocytes

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Exposure of adipocytes of rats to CdCl_2 caused acceleration of $[3\text{-}^3\text{H}]\text{glucose}$ incorporation into lipid maximally at $500\text{ }\mu\text{M}$ in Krebs–Ringer bicarbonate buffer, pH 7.4, containing 0.2% albumin. T.l.c. of the lipids extracted from adipocytes showed that Cd^{2+} increased labelling of di- and tri- $[^{14}\text{C}]\text{acylglycerols}$ predominantly. With increasing concentrations of glucose the apparent K_m value was not affected by Cd^{2+} , but the V value was increased, similarly to the effect of insulin. In the presence of insulin, Cd^{2+} ($5\text{ }\mu\text{M}$) exerted a consistent additive effect with a stimulatory effect of insulin on lipogenesis at all concentrations of insulin tested ($5\text{--}50\text{ }\mu\text{units/ml}$). The stimulation was observed at a high concentration of glucose, suggesting that Cd^{2+} accelerated intracellular metabolism of glucose, mimicking insulin. However, although Zn^{2+} and Mn^{2+} stimulated the transport at a rate similar to that observed with insulin ($200\text{ }\mu\text{units/ml}$), Cd^{2+} had no stimulating effect on the membrane transport of 3-*O*-methylglucose. The biological potency of Cd^{2+} and the insulin-like effects of Zn^{2+} , both of which metals belong to the same group in the Periodic Table, are similar towards glucose metabolism, but quite different towards glucose transport.

Cadmium is recognized to be a toxic non-essential trace element inhibiting numerous enzymes with functional thiol groups (Vallee & Ulmer, 1972). However, cadmium, like zinc (which is an essential trace element), belongs to group IIB of the Periodic Table and has chemical properties similar to those of zinc. Indeed, cadmium has been reported to function in proteins in place of zinc (Vallee & Ulmer, 1972; Davis & Avram, 1978; Andersson *et al.*, 1981). Moreover, cadmium was known to stimulate or inhibit some enzymes, depending on the ion concentration, such as mitochondrial ATPase (Houstek & Drahota, 1977; Rauchova & Drahota, 1979), glutathione peroxidase (Flegal *et al.*, 1980), δ -aminolaevulinic acid dehydratase, pyruvate dehydrogenase, malate dehydrogenase, glucose-6-phosphate dehydrogenase and acid phosphatase (Vallee & Ulmer, 1972).

In the course of preliminary investigations in regard to the role of trace metals on the function of the membrane and glucose metabolism in rat adipocytes, we noticed that CdCl_2 produced an insulin-like enhancement in the conversion of glucose into CO_2 and lipid, like the stimulation effect of other bivalent cations, i.e., zinc, manganese, nickel and mercury (Yamamoto *et al.*, 1983).

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There have been some investigations of the effects of zinc(II) (Coulston & Dandona, 1980; May & Contoreggi, 1982), manganese(II) (Rennie & Upton, 1981) or nickel(II) (Saggerson *et al.*, 1976) on glucose metabolism, in which studies those metal ions showed some actions mimicking those of insulin. The present paper demonstrates that the bivalent cadmium ion really acts as a stimulant of glucose intracellular metabolism in rat adipocytes, comparing the results of lipogenesis with those of zinc(II), manganese(II), nickel(II) or insulin.

Materials and methods

Chemicals

Bovine serum albumin (fraction V) was obtained from Armour Pharmaceutical Co. (Kankakee, IL, U.S.A.), and dialysed against Krebs–Ringer bicarbonate buffer (pH 7.4) with half the suggested concentration of Ca^{2+} (Rodbell, 1964). Monocomponent Actrapid pig insulin was purchased from Novo, Copenhagen, Denmark. CdCl_2 , MnCl_2 , NiCl_2 and ZnCl_2 (analytical grade) were obtained from Wako Chemical Co., Osaka, Japan, and used as trace-metal ions. Crude *Clostridium histolyticum* collagenase (type 1) was obtained from Worthing-

ton Biochemical Corp., Freehold, N.J., U.S.A. Cytochalasin B and 3-*O*-methylglucose were purchased from Sigma. D-[U-¹⁴C]Methylglucose and Hyamine hydroxide were obtained from Amersham/Searle Corp., and D-[3-³H]glucose was obtained from New England Nuclear.

Isolation of adipocytes

Adipocytes were prepared from the epididymal fat-pads of fed (*ad libitum*) male Wistar rats (120–160 g) by the collagenase digestion method of Rodbell (1964) with minor modifications. Cells were resuspended in Krebs–Ringer bicarbonate buffer containing 0.2% albumin and left for 30 min at 37°C before the incubation, to obtain a constant low baseline value (Vega & Kono, 1979; Yamamoto *et al.*, 1983).

Assay of glucose conversion into lipid

Adipocytes (4×10^4 cells) were incubated at 37°C for 90 min in Krebs–Ringer bicarbonate buffer containing 0.2% albumin and 0.5 mM-[3-³H]glucose (0.1 μ Ci), with or without additions, and were gassed with O₂/CO₂ (19:1) in a volume of 0.5 ml. Incubations were terminated by the addition of 0.25 ml of 2M-H₂SO₄.

The lipid material in the medium and the cells was extracted with toluene-based scintillant by the method described by Coulston & Dandona (1980). This lipid/toluene phase was washed with water, and the ³H radioactivity was counted. The result of this simple procedure was compared with that obtained by an established method for lipid, namely extraction with the solvent system chloroform/methanol/2M-KCl (see below). Very similar results were obtained in both methods, proving the reliability of the former.

3-O-Methylglucose transport

The standard assay mixture contained 0.05 mM-3-*O*-[¹⁴C]methylglucose (0.5 μ Ci) and fat-cells (2×10^5 – 2.5×10^5) in a final volume of 0.38 ml of Krebs–Ringer bicarbonate buffer containing 0.2% albumin. A mixture (0.36 ml, except for 3-*O*-methylglucose) was preincubated in the presence or absence of either metal ions and/or cytochalasin B (final concn. 50 μ M, added to correct for non-specific transport), at 37°C for 15 min and the transport was started by the addition of 3-*O*-methylglucose. After 10 s vigorous shaking at room temperature, the transport was terminated by the addition of 3 ml of cold Krebs–Ringer bicarbonate buffer containing 0.1% albumin and 0.3 mM-phloretin, followed by immediate filtration through Whatman GF/C glass microfibre paper. The filter paper was washed with 3×4 ml of cold

0.9% NaCl containing 0.1% albumin and dried in a scintillation-counting vial, as described by Czech (1976, 1980).

Lipid extraction and separation by t.l.c.

Adipocytes (1.8×10^4) were incubated at 37°C for 90 min with 0.5 μ Ci of [U-¹⁴C]glucose (0.5 mM) and additions, in 50 μ l of Krebs–Ringer bicarbonate buffer containing 0.2% albumin. Incubations were stopped by adding 1.5 ml of chloroform/methanol (1:2, v/v) (Billah *et al.*, 1979). The phases were split by adding 1.5 ml of chloroform and 0.6 ml of 2M-KCl solution, and the upper water phase was aspirated off. The chloroform phase was washed twice with methanol/1M-KCl (1:1, v/v), and a portion (50 μ l) of the washed extracts from each incubation mixture was dried in a counting vial, and redissolved with 0.15 ml of Hyamine hydroxide. The radioactivity was determined and the result was compared with that obtained by the extraction with toluene-based scintillant. Another portion (1.6 ml) of the residual extract from each tube was evaporated to dryness in a rotary evaporator under reduced pressure. The dried lipids were redissolved in chloroform/methanol (1:1, v/v), and applied on t.l.c. plates of Kieselgel 60 with concentrating zone (Merck), for neutral-lipid separation (solvent: light petroleum (b.p. 30–60°C)/diethyl ether/acetic acid, 80:30:1, by vol.).

The lipids were detected by exposure to iodine vapour and radioautographs were developed on Fuji X-Ray Film. The spots were identified by comparison with those of standard substances, and the radioactivity was determined in toluene/Triton X-100-based scintillation liquid (Patterson & Greene, 1965).

Radioactivity assay

Radioactivity was assayed in a Packard model 703 liquid-scintillation system by using a toluene-based scintillator containing 2,5-diphenyloxazole (4g) and 2,4-bis-(5-phenyloxazol-2-yl)benzene (50mg) per litre unless otherwise stated.

Results

Stimulation of lipogenesis

Investigations of the concentration-dependence of the lipogenesis from glucose on Cd²⁺ are shown in Fig. 1. In addition the effects of Zn²⁺, Mn²⁺ and Ni²⁺ were further investigated, since they have been reported to influence glucose metabolism in fat-cells. Mn²⁺ and Ni²⁺ are again thought to antagonize the action of Ca²⁺ in the insulin regulation of sugar transport in the diaphragm (Bihler,

Fig. 1. Dose-response relationship for the effects of Cd^{2+} and other bivalent metal ions on $[3\text{-}^3\text{H}]\text{glucose}$ incorporation into lipids with (b) or without (a) insulin ($5\text{ }\mu\text{units/ml}$). Assay conditions were as described in the text, with indicated concentrations of metal chloride: Δ , Cd^{2+} ; $\circ$, Zn^{2+} ; $\bullet$, Mn^{2+} ; $\blacktriangle$, Ni^{2+} . The results are means $\pm$ S.D. for three or more determinations and are expressed as percentages of control values in the absence of metal chloride in each experiment. The basal value without insulin was $4.11 \pm 0.44\text{ nmol}$ of glucose converted into lipid/ 4×10^4 cells, from eleven separate experiments. The response to insulin alone ($5\text{ }\mu\text{units/ml}$) was $234 \pm 42.9\%$ compared with basal values (from six separate experiments).

1972). Cd^{2+} is also an antagonist of Ca^{2+} . Cd^{2+} stimulated the apparent rate of glucose conversion into lipids, with maximal stimulation to more than twice the basal values achieved at $500\text{ }\mu\text{M}\text{-Cd}^{2+}$. Cd^{2+} and Zn^{2+} were almost equally effective on lipogenesis, in the magnitude of the stimulation and their effective concentrations, as shown in Fig. 1(a).

Lipogenesis from glucose was stimulated by either Mn^{2+} or Ni^{2+} , and maximally to about 2.5 times the values at 5 mM , a concentration one order of magnitude higher than that of Cd^{2+} or Zn^{2+} ($500\text{ }\mu\text{M}$) for maximal stimulation. Both maximum stimulations by Mn^{2+} and Ni^{2+} remained constant up to 10 mM metal ions, the highest concentration tested.

When adipocytes were incubated with combination of Cd^{2+} and $5\text{ }\mu\text{units/ml}$ of insulin, a further significant increase (20%) in lipogenesis occurred at concentrations of $5\text{ }\mu\text{M}\text{-Cd}^{2+}$ (Fig. 1b). At this concentration scarcely any stimulation was observed when Cd^{2+} alone was added. In the presence of insulin, however, the behaviour of Zn^{2+} was quite different from that of Cd^{2+} . Zn^{2+} increased the insulin-stimulated conversion rates at concentrations of $100\text{--}500\text{ }\mu\text{M}$, at which Zn^{2+} alone exhibits stimulation in a degree significantly similar to that obtained by Coulston & Dandona (1980). At concentrations above 3 mM , Mn^{2+} showed apparent additive effects to stimulation by insulin of lipid production, but no stimulation was apparent with Ni^{2+} at the concentrations tested ($5\text{ }\mu\text{M}\text{--}10\text{ mM}$).

Glucose-concentration dependence

Fig. 2 shows the glucose-concentration dependence of basal and Cd^{2+} - or insulin-stimulated $[3\text{-}^3\text{H}]\text{glucose}$ incorporation into lipids. Increasing the glucose concentration in the medium to the maximal value of 25 mM stimulated the formation of lipid from glucose. Cd^{2+} and insulin caused further increases, depending on the glucose concentration. The Lineweaver-Burk representations of all these saturation curves gave a linear relationship over the glucose-concentration range examined (results not shown). The apparent K_m value for glucose was determined to be $3.03 \pm 0.35\text{ mM}$ for lipid formation for basal conditions, with Cd^{2+} , and with insulin, whereas for V , the values in control conditions, with Cd^{2+} , and with insulin were 26.53 ± 1.01 , 41.87 ± 2.87 , and $61.57 \pm 6.57\text{ nmol}$ of glucose converted into lipid in $90\text{ min}/4 \times 10^4$ cells respectively (means $\pm$ S.E.M., $n = 3$). Although for each experiment different values for both K_m and V were obtained, in no case was K_m affected by Cd^{2+} or insulin, but the V was significantly increased ($P < 0.01$).

Fig. 2. Effects of glucose concentration on $[3\text{-}^3\text{H}]\text{glucose}$ incorporation into lipids

Assays were as described in the text except that the concentration was varied as indicated. Symbols: ●, basal; △, with Cd^{2+} (500 μM); ○, with insulin (5 $\mu\text{units/ml}$). The results are means $\pm$ S.D. for three or more determinations.

Time course

The rate of $[3\text{-}^3\text{H}]\text{glucose}$ incorporation into lipid was linear for 2 h in the absence or presence of Cd^{2+} (500 μM) or insulin (5 $\mu\text{units/ml}$) under the conditions tested. Cd^{2+} increased the insulin-stimulated rate constantly when it was added at a concentration of 5 μM with insulin (5 $\mu\text{units/ml}$) to the reaction medium. Prolonged incubation over 2 h occasionally caused a decrease in the control rate, but in the presence of Cd^{2+} and/or insulin stimulation remained linear until 3 h, the longest period tested.

Insulin dose-response relationship for effect of 5 μM - Cd^{2+}

Half-maximal stimulation of glucose incorporation into lipid occurred at an insulin concentration of 13.5 $\mu\text{units/ml}$, and at 12.0 $\mu\text{units/ml}$ in the presence of Cd^{2+} , i.e. at the same concentration. Cd^{2+} consistently stimulated (18–22% up) the conversion of glucose into lipids in the presence of insulin concentrations up to 50 $\mu\text{units/ml}$, the highest concentration tested. The effects of Cd^{2+} were statistically significant at 5, 10, 25 and 50 μunits of insulin/ml ($P < 0.05$; $n = 6\text{--}7$).

Table 1. Effect of bivalent metal ions on 3-O-methylglucose transport

Assays were described in the text with or without the indicated addition. The results are means $\pm$ S.D. for three or more determinations. Similar results were obtained in three or four separate experiments. * indicates significant difference from the control ($P < 0.05$).

Condition	Specific transport	
	(pmol/ 10^6 cells)	(%)
Control	27.4 ± 3.51	100
+ CdCl_2 (500 μM)	27.5 ± 4.77	99.1
+ ZnCl_2 (500 μM)	$38.5 \pm 4.65^*$	166.2
+ MnCl_2 (3 mM)	$43.5 \pm 3.66^*$	192.7
+ NiCl_2 (3 mM)	28.7 ± 5.90	104.7
+ Insulin (200 $\mu\text{units/ml}$)	$41.9 \pm 1.93^*$	183.5

Fig. 3. Time course of 3-O-methylglucose transport. Transport assays were as described in the text, except that the incubation period was varied as indicated. Symbols: ●, basal; □, with cytochalasin B; △, with Cd^{2+} (500 μM); ○, with insulin (200 $\mu\text{units/ml}$). The results are means $\pm$ S.D. for three or more determinations. Similar results were obtained in three separate experiments.

3-O-Methylglucose transport

Glucose transport by a saturable, carrier-mediated, diffusion was assayed by measuring the rapid kinetics of equilibration of the non-metabolizable analogue 3-O- $[^{14}\text{C}]\text{methylglucose}$ (Table 1). Insulin and Zn^{2+} stimulated this rate, as expected. Similar stimulation was also achieved in the presence of Mn^{2+} , increasing the rate to values similar to those found with insulin or Zn^{2+} . However, the virtual absence of effect of Cd^{2+} on 3-O-methylglucose transport was observed at a concentration of 500 μM (also see Fig. 3). At this concentration Cd^{2+}

stimulates lipid formation from glucose maximally. Results similar to those obtained with Cd^{2+} were obtained with Ni^{2+} .

Increased labelling of tri- and di-[^{14}C]acylglycerols

T.l.c. showed that tri- and di-acylglycerols were the main components of the increased lipid production through the stimulation of both insulin and Cd^{2+} , with minor increases in phospholipid, fatty acid, cholesterol, monoacylglycerol and other unknown components. For triacylglycerols, the radioactivity incorporated from [$\text{U-}^{14}\text{C}$]glucose of controls, with Cd^{2+} , and with insulin was 24347 ± 906 , 42035 ± 2750 and 66718 ± 6229 d.p.m./ 1.8×10^4 cells respectively, and for diacylglycerol, the values for controls, with Cd^{2+} , and with insulin were 6859 ± 569 , 26467 ± 2530 and 34407 ± 3221 d.p.m./ 1.8×10^4 cells respectively (means $\pm$ S.E.M., $n = 3$).

Discussion

The insulin-like effect of Cd^{2+} on the glucose utilization of the fat-cell has been shown. Cd^{2+} stimulated the conversion of glucose into lipids, especially tri- and di-acylglycerols, in low concentrations of an albumin-containing medium. We demonstrated previously that the low concentration of albumin (2 mg/ml of Krebs-Ringer bicarbonate) is a preferable incubation medium when the influence of a metal ion on the glucose metabolism in adipocytes is examined (Yamamoto *et al.*, 1983).

Caygill & Stein (1967) have reported that the apparent K_m was not affected by insulin, but V was increased, when [$\text{U-}^{14}\text{C}$]glucose conversion into $^{14}\text{CO}_2$ for 30 min of rat fat-cells was measured. In the study presented here, as with insulin, the apparent K_m for glucose was not affected by Cd^{2+} , and V was increased for lipid production from glucose, though the V value was lower than that with insulin (5 $\mu\text{units/ml}$) (Fig. 2). These results suggest that, although the K_m is thought to be the sum of several rate constants, one (or more) of which is sensitive to Cd^{2+} and insulin, each behaves as a non-competitive activator.

Since the demonstration by Crofford & Renold (1965) and Crofford (1967) that transport into rat cells appears to be the rate-limiting step in glucose metabolism, it has been generally assumed that agents which stimulate glucose utilization enhance transport. However, although this appears true for insulin, it has not been proved for most other agents known to stimulate fat-cell glucose oxidation or lipogenesis. May & Contoreggi (1982), using cytochalasin B as a specific inhibitor for

membrane transport, have shown that Zn^{2+} stimulates both 3-*O*-methylglucose transport and glucose metabolism to CO_2 , or to acylglycerol fatty acid and glycerol independently. In the presence of 25 mM-glucose, 500 μM - Cd^{2+} gave about 50% stimulation of adipocyte lipogenesis (Fig. 2) in a similar manner to Zn^{2+} . At 25 mM-glucose, intracellular metabolism and not transport is rate-limiting (Crofford & Renold, 1965). Therefore the persistent stimulation by Cd^{2+} indicates acceleration of an intracellular metabolism. However, this metal ion did not reveal any acceleration of membrane transport of the non-metabolizable sugar, 3-*O*-methylglucose, whereas insulin, Zn^{2+} and Mn^{2+} stimulated it (Table 1, Fig. 3).

It is known that Cd^{2+} interacts with phosphatidylethanolamine and phosphatidylserine monolayers, which are main components of cell membranes, and is effective in expanding them in water or in dilute NaCl solutions (Suzuki & Matsushita, 1968). However, from the results obtained here in the presence of Cd^{2+} , no accumulation of 3-*O*-methylglucose appeared over control values in fat-cells, so that it is evident that the increase of lipogenesis is not caused by the increasing diffusion through the injured membrane induced by Cd^{2+} in the condition tested.

So little is known about the functional significance of Cd^{2+} . At present we do not know how Cd^{2+} acts on glucose metabolism in adipocytes. It could enter the cell and influence one or more of the steps of events directly, or it could act on the cell membrane, and a resulting mediator act as a second intracellular messenger. The problems deserve further investigation.

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