

# Stimulatory action of cycloheximide on glucose metabolism in the rat epididymal fat pad

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**Abstract** The action of cycloheximide on some parameters of glucose and lipid metabolism was studied *in vitro* in epididymal fat pads from fasted rats. Incubation of fat pads with cycloheximide (1  $\mu\text{g/ml}$ ) for 2 hours resulted in a two-fold increase in glucose uptake, glucose oxidation, incorporation of glucose into lipids, and reesterification of free fatty acid. The increase in glucose oxidation was evident in experiments in which [ $U\text{-}^{14}\text{C}$ ], [ $1\text{-}^{14}\text{C}$ ], or [ $6\text{-}^{14}\text{C}$ ]glucose was added to the media, but it was absent when the media were supplemented with pyruvate. Incorporation of glucose into glycogen and accumulation of lactate in the medium were not seriously modified by the presence of cycloheximide. The stimulatory effect of cycloheximide on incorporation of glucose into lipids was absent when insulin or cortisol was added to the medium. A cycloheximide-mediated increase in glucose uptake seems to be responsible for the subsequent changes in glucose metabolism, and would seem to be independent of an inhibition in protein synthesis; puromycin and actinomycin-D did not mimic the cycloheximide action on glucose incorporation into lipids.

**Supplementary key words** adipose tissue metabolism · glucose uptake · glucose incorporation into lipids · free fatty acid esterification

Cycloheximide (CHM) is an antibiotic widely used as an inhibitor of protein synthesis *in vivo* (1) and *in vitro* (2). In a previous paper (3) we reported that 2 hr after an intraperitoneal injection of CHM into fasted male rats, there was a 10-fold increase in the incorporation of  $^{14}\text{C}$  from  $^{14}\text{C}$ -labeled glucose into epididymal fat pad lipids. Other inhibitors of protein synthesis did not produce this effect on lipid metabolism.

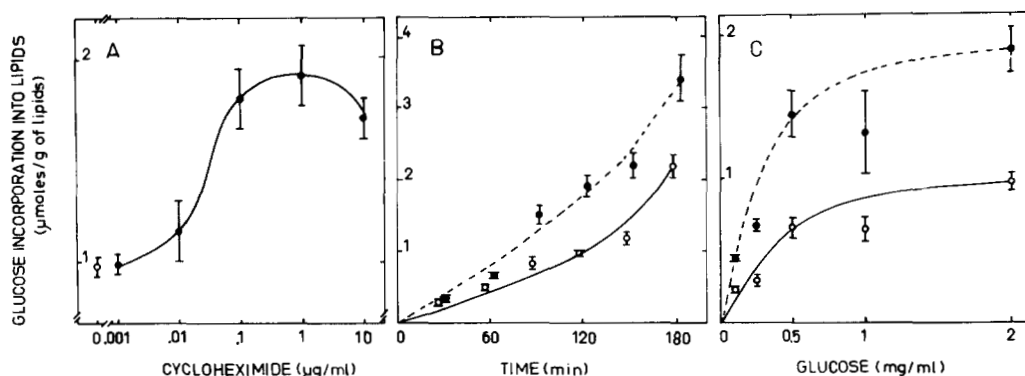
The aim of the present paper is to characterize the *in vitro* effects of CHM on adipose tissue metabolism in order to gain further insight on the mechanism of action of the antibiotic in a system that is less complex than the whole animal.

## MATERIALS AND METHODS

Cycloheximide, puromycin, bovine serum albumin (fraction V) and glycerokinase were obtained from Sigma Chemical Company (St. Louis, MO), Actinomycin-D was purchased from Nutritional Biochemical Corporation (Cleveland, OH).  $D\text{-}[U\text{-}^{14}\text{C}]$ Glucose (200 mCi/mmol),  $D\text{-}[1\text{-}^{14}\text{C}]$ glucose (5 mCi/mmol),  $D\text{-}[6\text{-}^{14}\text{C}]$ glucose (51.2 mCi/mmol) and  $L\text{-}[U\text{-}^{14}\text{C}]$ leucine (180 mCi/mmol) were obtained from International Chemical and Nuclear Corporation (Cleveland, OH). Hexokinase, glucose 6-phosphate dehydrogenase, and  $\alpha$ -glycerophosphate dehydrogenase were obtained from Behringer und Soehne (Mannheim, Germany).

Experiments were performed with male Wistar rats weighing between 120 and 170 g and fasted for 16–20 hr. Rats were killed by decapitation and exsanguinated. The epididymal fat pads were removed as fast as possible with minimal handling, rinsed in 0.85% NaCl, and incubated in a Dubnoff metabolic shaker at 37°C in stoppered 25-ml Erlenmeyer flasks. Usually one of the two tissues obtained from each animal served as control; the medium in which the contralateral tissue was incubated contained CHM. The amount of tissue per flask ranged between 120 and 150 mg ( $133.06 \pm 5.4$  mg control;  $138.77 \pm 1.96$  mg experimental; an average of 30 samples  $\pm$  SEM in each case). In addition to the tissue, each flask contained 3 ml of Krebs-Ringer bicarbonate buffer, pH 7.3, which contained: 115.38 mM NaCl, 4.74 mM KCl, 2.54 mM  $\text{CaCl}_2$ , 1.18 mM  $\text{KH}_2\text{PO}_4$ , 1.18 mM  $\text{MgSO}_4$ , 24.88 mM  $\text{NaHCO}_3$ , 150 mg of bovine serum albumin (fraction V, filtered through a millipore filter according to Dole (4) and containing

Abbreviations: CHM, cycloheximide; FA, fatty acids; FFA, free fatty acids; cyclic AMP, cyclic 3',5' adenosine monophosphate.



**Fig. 1.** Effect of cycloheximide on the incorporation of  $^{14}\text{C}$  from  $[^{14}\text{C}]$ glucose into lipids in epididymal fat pads. A) Dose-response, B) time-course and C) medium glucose concentration ( $[^{14}\text{C}]$ glucose specific activity was maintained constant). Panels B and C, control ( $\circ$ — $\circ$ ); CHM ( $\bullet$ — $\bullet$ ). Vertical lines represent the standard error of at least five independently incubated pads.

0.9  $\mu\text{eq}$  of FFA assayed by the method of Dole and Meinertz (5)), and 11.1 mM (2 mg/ml) glucose. In some experiments 1  $\mu\text{Ci}$  of  $^{14}\text{C}$ -labeled glucose was in the incubation medium. The incubation medium was heated to  $37^\circ\text{C}$  and gassed with 5%  $\text{CO}_2$ –95%  $\text{O}_2$  before the donor animals were killed, and the flasks were flushed with the same gas mixture after the tissue was added.

Lipids were extracted according to the method of Folch, Lees, and Sloane Stanley (6). In some experiments the lipid extracts were saponified and the distribution of radioactive carbon from glucose between glyceride-glycerol and fatty acids was studied by the method of Kornacker and Ball (7) adapted as previously described (3).

When lipolysis and esterification were studied, one pad from each rat was used to determine the initial concentration of glycerol and FFA, and the other one was incubated. The epididymal fat pads were homogenized in 3 ml of cold glass-distilled water. One ml each was taken from both medium and homogenates; FFA was determined by the method of Dole and Meinertz (5) and glycerol by the procedure of Wieland (8). The rate of FFA esterification was calculated according to Vaughan (9). Glucose uptake was measured as disappearance of hexose from the medium and was determined by the method of Slein (10).

Glycogen was quantified by the anthrone (11) method. The  $[^{14}\text{C}]$ glucose incorporated into glycogen was measured according to Hassid and Abraham (11). Lactic acid was measured by the method of Hohorst (12). Oxidation of radioactive glucose was measured by its conversion to  $^{14}\text{CO}_2$ , which was absorbed in 0.2 ml of 1 M Hyamine dissolved in methanol according to the method of Del Boca and Flatt (13). Protein synthesis was studied by measuring the incorporation of  $[^{14}\text{C}]$ leucine (0.2  $\mu\text{Ci}$  per flask) into protein.

Proteins were isolated according to Feigelson, Feigelson, and Fancher (14), and suspended in formic acid for measurement of radioactivity. The  $^{14}\text{C}$  radioactivity was measured in a Packard Tri-Carb liquid scintillation spectrometer (Packard Instrument Co., Downers Grove, IL) as previously described (3). Special conditions used in some particular experiments are given in the figures, tables, or in the text.

The results are presented as per gram of lipid or wet weight of tissue. Both measurements are comparable since it was found that under our experimental conditions  $70.69 \pm 2.23\%$  of the wet weight of the pads was lipid (mean  $\pm$  SEM of 11 determinations). Statistical comparisons between groups of data were performed by the Student's *t* test.

## RESULTS

**Fig. 1** shows the results of preliminary studies that defined experimental conditions used in subsequent incubations. CHM increased incorporation of  $[^{14}\text{C}]$ glucose into lipids at doses between 0.1  $\mu\text{g}/\text{ml}$  and 10  $\mu\text{g}/\text{ml}$  ( $P < 0.001$ ) (panel A, Fig. 1); a dose of 1  $\mu\text{g}/\text{ml}$  was selected for all subsequent studies including those of panels B and C. The incorporation of  $[^{14}\text{C}]$ glucose into lipids did not follow a linear pattern either in control or in CHM-treated tissues (panel B, Fig. 1). There was a lag period in the stimulatory action of CHM and the highest CHM/control ratio was obtained after 2 hr of incubation; this was the incubation time used for all the experiments including those represented in panels A and C. The maximum response to CHM in vivo was also found 2 hr after its administration (3).

The effect of increasing glucose concentration on the conversion of  $[^{14}\text{C}]$ glucose to lipids followed a

simple saturation kinetics (panel C, Fig. 1). The difference between control and CHM-treated tissues was statistically significant in all cases. A saturating glucose concentration (2 mg/ml) was used for all the experiments.

### Modifications of the incubation medium

The concentrations of several components of the incubation mixture were modified in an effort to magnify the action of the antibiotic and to obtain the optimal conditions to study its action.

It has been reported that glucose uptake by epididymal fat pads is related to the concentration of FFA in the medium (15) and that the mobilization of FFA is related to the calcium concentration in the extracellular fluids (16). In our system, the incubation mixture contained 50 mg/ml of albumin and 0.3  $\mu$ eq/ml of FFA. In some experiments the concentration of both substances was changed independently: albumin concentrations of 12.5–75 mg/ml, and FFA concentrations of 0–0.6  $\mu$ eq/ml were used. The calcium concentration usually (2.54 mM) was also modified in a series of experiments from 0 to 5.8 mM. Changes in the concentrations of these components in the incubation mixture did not modify the CHM-mediated stimulation of lipid metabolism (data not shown).

### Additions to the incubation medium

Some actions of CHM have been correlated to hormones (3, 17, 18). Thus, the effect of CHM on lipid metabolism reported in vivo was substantially decreased by orchietomy or adrenalectomy (3).

TABLE 1. Influence of cycloheximide on the incorporation of radioactive glucose into lipids in epididymal fat pads incubated with serum from control or cycloheximide-injected rats

| Addition                                       | Control                             | CHM                    | CHM/<br>Control | P      |
|--|-------------------------------------|------------------------|-----------------|--------|
| <i><math>\mu</math>moles/g of lipids</i>       |                                     |                        |                 |        |
| None   | 0.98 $\pm$ 0.06 <sup>a</sup><br>(8) | 1.91 $\pm$ 0.16<br>(6) | 1.95            | <0.001 |
| Serum <sup>b</sup> from saline-treated animals | 0.74 $\pm$ 0.08<br>(3)              | 1.20 $\pm$ 0.12<br>(3) | 1.62            | <0.05  |
| Serum <sup>b</sup> from CHM-treated animals    | 1.77 $\pm$ 0.10<br>(3)              | 2.65 $\pm$ 0.07<br>(3) | 1.50            | <0.005 |

<sup>a</sup> The results are expressed as the mean  $\pm$  SEM with the number of observations in parentheses.

<sup>b</sup> Serum was obtained from a blood sample collected 2 hr after the administration of saline or cycloheximide dissolved in saline at a dose of 1 mg/kg of body weight. Each flask containing the incubation mixture described in Materials and Methods was supplemented with 0.15 ml of serum.

TABLE 2. Effects of cycloheximide on lipolysis and free fatty acid reesterification

|               | Rate of Lipolysis<br>(Glycerol<br>Production) | Net Change<br>in FFA                      | Rate of<br>Esterification                      |
|---------------|---|---|--|
|               | <i><math>\mu</math>mole/g wet weight</i>      | <i><math>\mu</math>eq/g wet weight</i>    | <i><math>\mu</math>eq FFA/g<br/>wet weight</i> |
| Control       | 4.94 $\pm$ 0.20 <sup>a</sup><br>(7)           | 11.66 $\pm$ 0.98<br>(7)                   | 3.16   |
| Cycloheximide | 6.65 $\pm$ 0.75<br>(7)<br><i>P</i> < 0.05     | 13.66 $\pm$ 1.71<br>(7)<br><i>P</i> < 0.3 | 6.29   |

<sup>a</sup> Mean  $\pm$  SEM with the number of observations in parentheses.

Because of this, several hormones were tested for their ability to magnify the CHM effect.

Epinephrine (10  $\mu$ g/ml), corticosterone-21-acetate (0.1–1 mg/ml) and testosterone (0.01–0.1 mg/ml) added to the incubation medium did not affect the response to CHM. However, in the presence of cortisol (1 mg/ml) or insulin (1 mU/ml) the effect of CHM was not observed (results not shown).

In the presence of serum from saline-treated animals, the conversion of [<sup>14</sup>C]glucose into lipids was decreased in the epididymal fat pads incubated either with or without CHM. Opposite results were observed when serum from rats treated with CHM was used (Table 1). Of interest is the response obtained in experiments in which the medium was supplemented with CHM and with serum from CHM-treated rats. The higher incorporation obtained in these pads cannot be attributed to an extra dose of CHM, since an optimal dose was used (panel A, Fig. 1). Additional volumes of serum produced results quite similar to those reported in Table 1 (results not shown).

### Lipolysis and reesterification

The action of CHM on lipolysis and reesterification was investigated to determine the metabolic pathway

TABLE 3. Effects of cycloheximide on glucose metabolism

|   | Control                              | CHM                      | CHM/<br>Control | P      |
|---|--------------------------------------|--------------------------|-----------------|--------|
| <i><math>\mu</math>moles/g wet weight</i> |                                      |                          |                 |        |
| Glucose uptake                            | 3.03 $\pm$ 0.31 <sup>a</sup><br>(11) | 6.78 $\pm$ 0.41<br>(12)  | 2.24            | <0.001 |
| Glucose incorporation into glycogen       | 0.067 $\pm$ 0.014<br>(6)             | 0.102 $\pm$ 0.026<br>(6) | 1.52            | <0.3   |
| CO <sub>2</sub> formation                 | 1.04 $\pm$ 0.11<br>(5)               | 1.82 $\pm$ 0.24<br>(6)   | 1.75            | <0.01  |

<sup>a</sup> Mean  $\pm$  SEM. The number of observations is in parentheses.

TABLE 4. Relative distribution of radioactive carbon from glucose in different fractions of lipid extracts of epididymal fat pads

| Additions             | Nonsaponifiable Lipids   | %           |                    |
|-----------------------|--------------------------|-------------|--------------------|
|                       |                          | Fatty Acids | Glyceride-Glycerol |
| None (4) <sup>a</sup> | 4.35 ± 1.30 <sup>b</sup> | 5.60 ± 1.05 | 88.97 ± 2.14       |
| CHM (5)               | 2.86 ± 1.63              | 9.18 ± 3.32 | 89.66 ± 3.36       |

<sup>a</sup> Number of experiments.

<sup>b</sup> Mean ± SEM.

that was preferentially stimulated by the antibiotic. CHM enhanced the net production of glycerol by 34.6% and that of FFA by 17.1% (Table 2). The rate of reesterification was calculated from the data of the table. Twice as much FFA was esterified in pads incubated with CHM. Similar results had been previously obtained in vivo (3).

### Effect of CHM on glucose metabolism

A general study of the influence of CHM on glucose metabolism in the epididymal fat pads was carried out. CHM mainly stimulated both glucose uptake and glucose oxidation (Table 3). The magnitude of stimulation was parallel to the increase in the conversion of [<sup>14</sup>C]glucose into lipids (Fig. 1). A slight but statistically insignificant increase in glucose incorporation into glycogen was also noted.

Since the differential response in glucose metabolism shown in Table 3 could have been due to utilization of endogenous glycogen or to the release of lactate into the incubation mixture, both of these parameters were examined. After 2 hr of incubation, the glycogen content decreased from 2.11 ± 0.22 μmoles of glucose/g wet weight to 0.66 ± 0.06 in control pads and to 1.00 ± 0.11 when the antibiotic was added to the medium (mean ± SEM of 4,6, and

TABLE 5. Effect of cycloheximide on CO<sub>2</sub> formation from glucose

| <sup>14</sup> C-Labeled Glucose | Pyruvate 5 mM | Control                         |                    | CHM  | CHM/Control | P |
|---------------------------------|---------------|---------------------------------|--------------------|------|-------------|---|
|                                 |               | μmoles/g wet weight             |                    |      |             |   |
| [1- <sup>14</sup> C]            | -             | 0.73 ± 0.08 <sup>a</sup><br>(6) | 1.41 ± 0.16<br>(5) | 1.93 | <0.005      |   |
|                                 | +             | 0.80 ± 0.07<br>(6)              | 0.96 ± 0.06<br>(6) | 1.20 | <0.1        |   |
| [6- <sup>14</sup> C]            | -             | 0.52 ± 0.06<br>(6)              | 0.84 ± 0.10<br>(6) | 1.62 | <0.05       |   |
|                                 | +             | 0.21 ± 0.03<br>(5)              | 0.25 ± 0.03<br>(6) | 1.19 | <0.3        |   |

<sup>a</sup> Mean ± SEM. The number of observations is in parentheses.

6 determinations respectively). Lactate accumulation in the medium was equivalent in the absence or presence of CHM (control 4.73 ± 0.62 μmoles/g and CHM-treated 4.40 ± 0.54 μmoles/g; mean ± SEM of 6 determinations in each case).

Table 4 shows data on the effect of CHM on conversion of [<sup>14</sup>C]glucose to lipid in the epididymal fat pads. Twice as much radioactivity was present in lipid extracts from tissues treated with CHM, but the relative distribution was not affected.

In order to determine if the stimulation of glucose oxidation by CHM (Table 3) increased Krebs cycle activity or pentose phosphate cycle activity, experiments with [1-<sup>14</sup>C]glucose and with [6-<sup>14</sup>C]glucose were performed. In both cases the stimulatory action of CHM on hexose oxidation was present (Table 5). The addition of pyruvate masked the action of the antibiotic, suggesting that the stimulation of oxidation occurred via the Krebs cycle.

### Inhibition of protein synthesis

An attempt was made to correlate the inhibition of protein synthesis due to CHM with its effect on lipid metabolism in epididymal fat pads. Other inhibitors of protein synthesis, such as actinomycin-D and puromycin, did not share with CHM the ability to increase the conversion of glucose into lipids of the fat pads (Fig. 2). Furthermore, puromycin decreased this process in agreement with the report of Fain (19).

## DISCUSSION

Our present results resemble the in vivo effect of CHM on epididymal fat pads (3). The qualitative

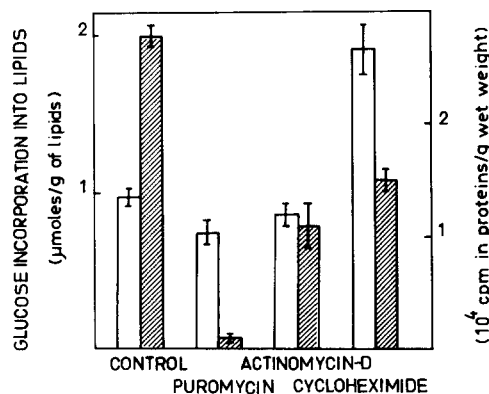


Fig. 2. Effect of several inhibitors of protein synthesis on the incorporation of [<sup>14</sup>C]glucose into lipids (open bars) and [<sup>14</sup>C]leucine into proteins (cross hatched bars). Doses: puromycin 10<sup>-4</sup> M, actinomycin-D 10<sup>-7</sup> M, and cycloheximide 3.55 × 10<sup>-6</sup> M (1 μg/ml). Vertical lines represent the standard error of at least five independently incubated pads.

nature of the response to CHM was similar in vivo and in vitro in the following aspects: *a*) most of the label from glucose was in the glyceride-glycerol moiety of the lipid extracts (Table 4); *b*) the actions on lipolysis and reesterification (Table 2); *c*) the time required to show a maximum effect (Fig. 1, Panel B); and *d*) the absence of correlation with an inhibition in protein synthesis (Fig. 2). Nevertheless, the quantitative stimulation in conversion of glucose into lipids produced by the antibiotic was increased 10-fold in vivo (3) and only 2-fold in vitro (Fig. 1).

Some humoral factor appears to be involved in the effect of CHM on lipid metabolism in adipose tissue. A further increase in the incorporation of glucose into lipids was detected when serum from CHM-treated animals was added to the incubation mixture containing the optimal dose of the antibiotic (Table 1). In addition, some actions of CHM have been shown to be hormone-mediated (17, 18). Thus the 10-fold increase in the incorporation of glucose into lipids of the epididymal fat pads from male rats fasted for 16–20 hr was lower in orchietomized animals (2-fold), adrenalectomized animals (3-fold), and in the parametrial adipose tissue from female rats (4.6-fold) (3). Therefore the differences between our results and those of other authors who have studied the effect of CHM on adipose tissue under different experimental conditions are not surprising.

Fain (2) and Caldwell and Fain (20), in studies with isolated fat cells from starved female rats, and Goodman (21), in studies with epididymal fat pads from hypophysectomized, fed male rats, reported no change in the basal rates of glycerol and FFA production due to CHM.

The CHM-inhibition of lipid metabolism in rat adipose tissue reported by Jomain-Baum and Hanson (22) is not related to the present finding since these authors used the antibiotic at a dose 1000 times higher than that employed in the present study.

The increase in glucose uptake produced by the in vitro addition of CHM to epididymal fat pads might explain most of the other effects on glucose and lipid metabolism in the pads. The stimulation in glucose uptake was mainly reflected in glucose oxidation (Table 3) and FFA reesterification (Table 2). Glycogen synthesis, the hexose monophosphate shunt, and lactate accumulation in the medium were affected to a lesser extent.

The results in Table 5 suggest that there was an increased oxidation of the entire hexose molecule, presumably via the Krebs tricarboxylic acid cycle. Furthermore, the addition of pyruvate, in order to dilute glucose metabolites entering the tricarboxylic

acid cycle (15), masked the stimulatory action of the antibiotic (Table 5). The stimulation in glucose oxidation by CHM is consistent with the observed increase in fatty acid reesterification, since the latter process depends on an energy source (23) and enhances oxygen consumption (24).

The esterification of FFA depends on glucose concentration in the medium (9) and considering that the CHM action was observed at all the concentrations of glucose tested (Fig. 1, panel C), it might be assumed that the increase in reesterification produced by the antibiotic was a consequence of the enhanced glucose uptake. The pattern of distribution of label between fatty acids and glyceride-glycerol was not modified by CHM (Table 4) and is further evidence in favor of an increase in FFA reesterification. The basal rate of lipolysis was slightly stimulated by CHM (Table 2). This increase probably is related to the accumulation of cyclic AMP reported in isolated fat cells treated with the antibiotic (25). Experiments are in progress to elucidate the mechanism of these effects of CHM in adipose tissue. ■

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