Inhibition of Insulin-Stimulated Xylose Uptake in Rat Soleus Muscle by Cycloheximide

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Summary. Cycloheximide (20–200 mg/l) did not affect basal D-[U-¹⁴C]xylose uptake by rat soleus muscle ($2.4 \pm 0.2 \mu\text{mol} \cdot \text{g}^{-1} \cdot \text{h}^{-1}$). However, the stimulatory effect of insulin on sugar transport was progressively reduced from 375% above basal in control muscles to 170% in muscles exposed to 200 mg cycloheximide/l but above this concentration cycloheximide inhibited basal xylose uptake without further effect on the incremental effect of insulin. Cycloheximide affected the insulin dose-response curve both by depressing insulin sensitivity and by reducing the maximum stimulatory effect of the hormone. In contrast to the inhibition of insulin action, which increased progressively over the range 20–200 mg cycloheximide/l,

muscle protein synthesis was inhibited maximally at a concentration of 10 mg/l. Cycloheximide also inhibited the insulin-mimetic effects of anoxia, 2:4-dinitrophenol, salicylate, cooling, hydrogen peroxide, diamide, vitamin K₃, hyperosmolarity and EDTA, but did not affect concanavalin A-stimulated xylose uptake. It is concluded that cycloheximide inhibits insulin-stimulated sugar transport at some late post-receptor step, and that this effect of cycloheximide is not secondary to the inhibition of protein synthesis.

Key words: Sugar transport, insulin, cycloheximide, concanavalin A.

The overall process whereby insulin stimulates glucose transport in muscle and adipose tissue can be considered in three phases: (a) the initial interaction of the hormone with its receptor, (b) the signalling mechanism between the receptor and the glucose carrier, and (c) activation of the carrier itself. During the last decade the structure and function of the insulin receptor has been studied extensively [1, 2]. More recently, it has been suggested that activation of glucose transport involves the translocation of sugar carriers from intracellular sites to the plasma membrane [3, 4]. However, the nature of the signal which links the receptor to the glucose transport system has yet to be defined. Insulin promotes the release of a chemical mediator from the plasma membrane, which appears to mediate the effect of the hormone intracellularly on glycogen synthase and pyruvate dehydrogenase [5–7]. As yet this mediator has not been associated with insulin-stimulated sugar transport.

One approach to the study of the coupling system is the use of agents which inhibit insulin action at the post-receptor level. Previous reports from this laboratory have described the effects of two such agents, denerivation [8] and the divalent cation ionophore, A23187 [9], on insulin-stimulated sugar transport in soleus muscle. Yu & Gould [10] observed that the protein synthesis in-

hibitor, cycloheximide, also blocked insulin-stimulated sugar transport post-receptorally; thus, cycloheximide inhibited insulin action but did not affect ¹²⁵I-insulin binding. In this paper we present the results of further studies concerning the nature of the inhibitory effect of cycloheximide on sugar transport in soleus muscle.

Materials and Methods

Soleus muscles weighing approximately 30 mg were removed from Sprague-Dawley rats (70–90 g) fed ad libitum. Muscles were incubated at 37 °C under an atmosphere of O₂-CO₂ (95:5, v/v); anaerobic incubations were under N₂-CO₂ (95:5, v/v). The basic ('bicarbonate') medium contained NaCl (118 mmol/l), KCl (4.8 mmol/l), CaCl₂ (2.6 mmol/l), MgSO₄ (1.2 mmol/l), KH₂PO₄ (1.2 mmol/l) and NaHCO₃ (25 mmol/l); before use the medium was gassed with the appropriate gas mixture. Variations from this basic medium are detailed in the text.

Sugar Transport

Soleus muscles were first pre-incubated under the test conditions, xylose uptake was then determined using the method of Korbl et al. [11]. Briefly, this procedure measures intracellular xylose as the difference between the distribution of D-xylose and the extracellular marker, D-sorbitol. Muscles were incubated for 5 min at 37 °C in bicar-

Table 1. Effect of cycloheximide on activators of muscle sugar transport

Activator	Period of exposure to activator (min)	Xylose uptake ($\mu\text{mol} \cdot \text{g}^{-1} \cdot \text{h}^{-1}$)		Inhibition (%) [*]	<i>p</i>
		Activator	Activator and cycloheximide		
Anoxia	60 ^a	4.9 ± 0.4	3.6 ± 0.1	52	< 0.05
2 : 4-dinitrophenol (0.5 mmol/l)	10	7.0 ± 0.3	3.8 ± 0.2	70	< 0.005
Salicylate (20 mmol/l)	30	7.6 ± 0.6	4.8 ± 0.5	54	< 0.001
Cooling	180 ^b	7.9 ± 0.8	5.5 ± 0.4	44	< 0.01
H ₂ O ₂ (5 mmol/l)	10	6.2 ± 0.1	5.0 ± 0.3	32	< 0.05
Diamide (0.2 mmol/l)	30	5.8 ± 0.1	5.3 ± 0.2	15	< 0.05
Vitamin K ₅ (0.05 mmol/l)	30	7.4 ± 0.4	5.6 ± 0.3	36	< 0.01
Concanavalin A (500 mg/l)	30	6.5 ± 0.3	6.1 ± 0.2	10	< 0.30
Mannitol (200 mmol/l)	30	7.7 ± 0.4	6.0 ± 0.3	32	< 0.005
EDTA (5 mmol/l)	5 ^c	7.7 ± 0.4	6.4 ± 0.3	25	< 0.02

Except as noted below, soleus muscle pairs were incubated for 30 min at 37 °C under aerobic conditions ± cycloheximide (200 mg/l). The agents shown were then added and the incubations continued for the times shown in the second column. Values are mean ± SEM of five determinations: (a) Muscles were incubated for 60 min at 37 °C under 95% N₂: 5% CO₂; (b) After 30 min at 37 °C, muscles were incubated for 180 min at 0 °C; xylose uptake was determined at 37 °C; (c) The bicarbonate medium was modified by the omission of Ca²⁺ and Mg²⁺.

* calculated on the basis of the incremental stimulation above basal xylose uptake ($2.4 \pm 0.2 \mu\text{mol} \cdot \text{g}^{-1} \cdot \text{h}^{-1}$)

bonate medium (0.5 ml) containing D-[U-¹⁴C]xylose (10 mmol/l, spec. act. 0.03 $\mu\text{Ci}/\mu\text{mol}$) and D-[1-³H]sorbitol (10 mmol/l, spec. act. 0.1 $\mu\text{Ci}/\mu\text{mol}$). Muscles were solubilized by heating for 2 h at 50 °C in NCS tissue solubilizer (0.5 ml); radioactivity was determined by counting in the presence of a toluene scintillator solution.

Protein Synthesis

Muscles were incubated for 15 min at 37 °C in bicarbonate medium containing L-[U-¹⁴C]leucine (1 mmol/l, spec. act. 1 $\mu\text{Ci}/\mu\text{mol}$), then rinsed briefly in ice-cold water and homogenized in trichloroacetic acid (1 ml, 100 g/l). The homogenizer was rinsed twice, each with 1 ml of trichloroacetic acid (100 g/l); the combined homogenate and rinsings were heated for 5 min at 100 °C, cooled in ice and filtered through a 2.1 cm Whatman GF/A glass filter disc in a Millipore filter. The precipitate on the filter was washed three times each with 5 ml of ice-cold L-leucine (10 mmol/l) in trichloroacetic acid (50 g/l) then three times each with 1 ml of absolute ethanol and then dried for 2 min at 110 °C. The filter discs were placed into counting vials with 1 ml of NCS tissue solubilizer and the precipitate dissolved by heating for 2 h at 50 °C. Toluene scintillator (10 ml) was added and the radioactivity counted.

Materials

D-[U-¹⁴C]xylose, L-[U-¹⁴C]leucine and NCS tissue solubilizer were from Amersham (Australia) Sydney. D-[1-³H]sorbitol was from New England Nuclear Corporation, Boston. Beef insulin, twice recrystallized, was from the Commonwealth Serum Laboratories, Melbourne. 2:4-dinitrophenol and EDTA were from British Drug House Chemicals (Australia) Sydney. Concanavalin A was from Boehringer-Mannheim (Australia), Sydney. Cycloheximide and mannitol were from Calbiochem-Behring (Australia), Sydney. Vitamin K₅ was from Tokyo Kasei Kogyo, Tokyo. Diamide was from Sigma Chemicals, St. Louis, Mo. USA.

Statistical Analysis

To minimise the effect of biological variation between individual animals, wherever possible the experiments were designed using paired controls. One muscle from each pair was incubated under test conditions, while the second served as the control. The results of these experiments were analyzed for statistical significance using Student's *t*-test as applied to paired samples. Where it was not possible to use

paired controls, muscles taken from litter mates were distributed randomly among the experimental groups and the results subjected to statistical analysis using the standard Student's *t*-test.

Results

Soleus muscles were exposed to cycloheximide (20–1000 mg/l) for 60 min in the presence and absence of insulin (100 U/l). Insulin-stimulated xylose uptake was inhibited by cycloheximide over the range 20–200 mg/l (Fig. 1). When the cycloheximide concentration was increased beyond 200 mg/l, xylose uptake in insulin-treated muscles was depressed further; however, this seems to be related to the effect of the inhibitor on basal sugar transport, as the incremental stimulation by insulin was unchanged over the range 200–1000 mg/l. To avoid any complications associated with the inhibition of basal sugar transport, a concentration of 200 mg cycloheximide/l was used for subsequent experiments.

Fig. 1. Inhibition of insulin-stimulated xylose uptake by cycloheximide. Soleus muscle pairs were incubated for 60 min at 37 °C in bicarbonate medium ± insulin (100 U/l) in the presence of cycloheximide (0–1000 mg/l). Values are mean ± SEM of five determinations; ●—●, in the presence of insulin, ○—○, without insulin

Fig. 3. Effect of cycloheximide on protein synthesis by soleus muscle $\blacktriangle$ — $\blacktriangle$. Soleus muscles incubated for 60 min at 37°C in the presence of cycloheximide (0–200 mg/l). [U - 14 C] leucine (1 mmol/l, spec. act. 1 μ Ci/ μ mol) was added 15 min before the end of the incubation period. Values are mean $\pm$ SEM of five determinations. Insulin-stimulated xylose uptake values ($\bullet$ — $\bullet$) are from Figure 1.

The effect of cycloheximide on the insulin-sensitivity curve was examined over the range 0–100 U insulin/l. The results presented in Figure 2A show that cycloheximide reduced insulin-sensitivity; the inhibitor also seemed to lower the maximum response to the hormone. To obtain an objective (although not necessarily unbiased) estimate of the maximum response, the data from Figure 2A was replotted in the form of the double reciprocal plot (Figure 2B), from which the maximum rates of xylose uptake in insulin-treated muscles were calculated to be $11.0 \mu\text{mol} \cdot \text{g}^{-1} \cdot \text{h}^{-1}$ for control muscles and $7.7 \mu\text{mol} \cdot \text{g}^{-1} \cdot \text{h}^{-1}$ for muscles exposed to cycloheximide. Furthermore, the concentration of insulin required to stimulate sugar transport by 50% was increased fivefold, from 0.2 U/l in control muscles to 1.1 U/l in cycloheximide-treated muscles.

Since cycloheximide is a potent inhibitor of protein synthesis, it is pertinent to ask whether its effect on insulin-stimulated sugar transport is secondary to the inhibition of muscle protein synthesis. The effects of cycloheximide on insulin-stimulated xylose uptake and on the incorporation of L-[U - 14 C]leucine into muscle protein are compared in Figure 3. Protein synthesis was in-

Fig. 2 A and B. Effect of cycloheximide on insulin sensitivity. **A** Soleus muscle pairs were incubated for 60 min at 37°C $\pm$ cycloheximide (200 mg/l); insulin (0–100 U/l) was added 15 min before the end of the incubation period. Values are mean $\pm$ SEM of five determinations; $\circ$ — $\circ$, in the presence of cycloheximide $\bullet$ — $\bullet$, without cycloheximide **B** Double reciprocal plot of the data shown in Figure 2A. Incremental insulin stimulation was calculated as the difference between xylose uptake measured in the presence and absence of insulin

hibited 96% when muscles were treated with cycloheximide (10 mg/l); there was no further effect on protein synthesis above this concentration of cycloheximide. In contrast, the effect of the inhibitor on insulin action increased progressively over the range 20–200 mg/l.

To define the nature of the inhibitory effect of cycloheximide more precisely, we examined the effect of this inhibitor on the action of a number of other agents which have been shown to stimulate sugar transport in muscle: anoxia, 2:4-dinitrophenol, salicylate, cooling, hyperosmolarity (200 mmol mannitol/l), EDTA, H_2O_2 , diamide, vitamin K_5 and concanavalin A [8, 11]. Cycloheximide (200 mg/l) inhibited the stimulatory effects of all these agents with the sole exception of concanavalin A (Table 1). The inhibitory action of cycloheximide, calculated in terms of inhibition of the incremental effects of these agents over basal xylose uptake, varied from 15% inhibition (diamide) to 70% inhibition (2:4-dinitrophenol); by comparison, in the experiments shown in Figures 1 and 2, cycloheximide (200 mg/l) inhibited insulin-stimulated xylose uptake by 40–54%.

Discussion

During the course of studying the ATP-dependence of insulin-stimulated sugar transport in rat soleus muscle, Yu and Gould [10] observed that cycloheximide, a potent inhibitor of protein synthesis, also inhibited insulin-stimulated xylose uptake. Because it does not inhibit ^{125}I -insulin binding by soleus muscle, cycloheximide has the potential for use as a metabolic probe to study insulin action at the post-receptor level. We have confirmed that cycloheximide does not affect ^{125}I -insulin binding by soleus muscle, even at inhibitor concentrations as high as 1000 mg/l (J. R. Forsayeth, unpublished data). Up to a concentration of 200 mg/l, the inhibitory action of cycloheximide was restricted to insulin-stimulated sugar transport; above this concentration sugar transport was affected also.

It is now apparent that not all the effects of protein synthesis inhibitors on cellular metabolism are due to

the inhibition of protein synthesis [12–16]. On the basis of differences between the effects of cycloheximide and puromycin and because of the rapidity with which cycloheximide inhibited insulin-stimulated xylose uptake, Yu and Gould [10] concluded that this effect was not secondary to the inhibition of protein synthesis. We have found that higher concentrations of cycloheximide are required to inhibit insulin-stimulated xylose uptake than are needed to block completely that portion of protein synthesis which is sensitive to this inhibitor (96% of total). This observation is further evidence that the insulin-antagonistic effect of cycloheximide is independent of the inhibition of protein synthesis.

In contrast to the action of denervation, which had little effect on other insulinomimetic agents apart from insulin, cycloheximide inhibited the action of 9 of the 10 agents tested (Table 1), which places the site of action of this inhibitor closer to the glucose carrier than denervation. Three of the agents, anoxia, 2:4-dinitrophenol and salicylate, are believed to stimulate muscle sugar transport as a consequence of their lowering of ATP levels [11]. The effect of these three agents has been linked to that of cooling [17]. The inhibitory effect of cycloheximide on the action of these four agents (44–70% inhibition) was greater than on the other insulinomimetic agents (15–36% inhibition). Whether or not this apparent difference has significance in terms of the action of these various agents remains to be seen.

The only agent which appeared to be insensitive to inhibition by cycloheximide was concanavalin A. This finding is paralleled by the observation of Yasmeen et al. [13] that puromycin inhibited concanavalin A-stimulated sugar transport in thymocytes, but cycloheximide did not. Concanavalin A is one of a series of plant lectins which stimulate glucose transport in adipocytes [18, 19]. It has been suggested that the insulinomimetic action of concanavalin A could result from the binding of the lectin to the insulin receptor, thus provoking the release of the 'insulin' signal [20]. However, its insensitivity to cycloheximide, in contrast to the sensitivity of insulin and the other agents tested, suggests that its action cannot be concerned with the insulin receptor. Previous experiments, which showed that the effect of concanavalin A on muscle sugar transport was not attenuated by denervation, prompted a similar conclusion [8]. The present result modifies this observation by suggesting that the action of concanavalin A is localized at some point much closer to the carrier system, beyond the site of action of cycloheximide.

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