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EFFECT OF PROTEIN SYNTHESIS INHIBITORS ON XYLOSE UPTAKE AND ^{125}I-INSULIN BINDING BY RAT SOLEUS MUSCLE

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SUMMARY. Prolonged exposure (90-180 min) to cycloheximide (0.2 mg/ml), puromycin (0.2 mg/ml) or chloramphenicol (0.1 mg/ml) did not affect ^{125}I-insulin binding by rat soleus muscle. Chloramphenicol (2 mg/ml) depressed insulin binding and insulin-stimulated xylose uptake; these effects were attributed to the "toxic" effect of chloramphenicol on muscle ATP levels. Cycloheximide and puromycin inhibited insulin-stimulated xylose uptake without affecting ATP. Puromycin and chloramphenicol, but not cycloheximide, also inhibited basal sugar transport. This difference, and the rapid onset of all these inhibitory effects, suggest that they are not due to the inhibition of protein synthesis, but rather to some more direct effect on sugar transport itself.

Recent studies in this laboratory have shown that the stimulatory effect of insulin on sugar transport was progressively lost (1) and ^{125}I-insulin binding depressed (2) when rat soleus muscles were incubated under anaerobic conditions for longer than 30 min. These effects were associated with the depletion of muscle ATP, which suggested that there was some ATP-dependent step involved in the binding of insulin to its receptor. Alternatively, the role of ATP may be simply as a source of energy for protein (i.e. receptor) synthesis. To investigate this possibility, we have studied the effect of inhibitors of protein synthesis on the binding of ^{125}I-insulin. As reported below, insulin binding was not affected by the inhibition of protein synthesis for up to 3 hours; however, all of the inhibitors tested inhibited sugar transport by some mechanism which does not seem to involve their inhibition of protein synthesis.

METHODS

Soleus muscles weighing approximately 30 mg were obtained from Wistar rats (70-90 g). Following preincubation at 37° in Krebs-bicarbonate medium under the conditions described below, xylose uptake, ^{125}I-insulin binding and ATP were determined using methods previously described (1,2). For the deter-

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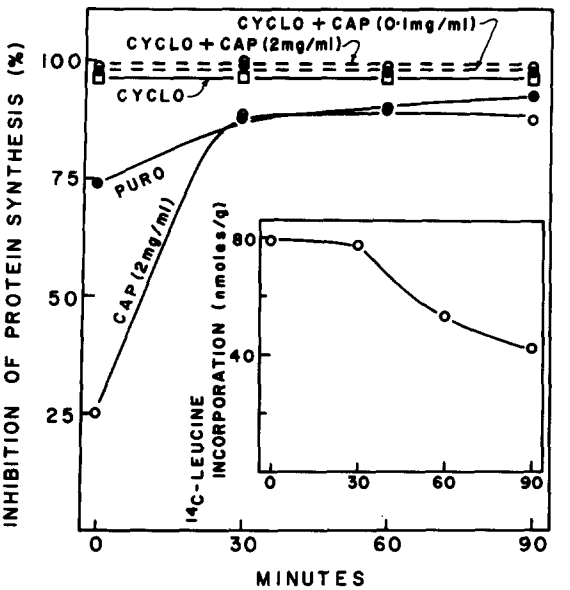

Figure 1. Effect of cycloheximide, puromycin and chloramphenicol on the incorporation of ^{14}C-leucine into muscle protein. Soleus muscles were pre-incubated for up to 90 min at 37° under O_2-CO_2 in 0.95 ml of Krebs-bicarbonate medium containing cycloheximide ("CYCLO", 0.2 mg/ml), puromycin ("PURO", 0.2 mg/ml) and/or chloramphenicol ("CAP", 0.1 or 2 mg/ml) as shown. [U-^{14}C] leucine (final conc. 1 mM sp. act. 1 µCi/µmol) was then added in a volume of 50µl and the incubation continued for a further 15 min. Trichloroacetic acid-precipitable radioactivity was then isolated and counted as described under METHODS. The effects of the inhibitors are shown as the percentage inhibition of protein synthesis measured in control muscles (shown in the inset). Each point is the mean of 3 determinations.

mination of protein synthesis the muscles were incubated for 15 min at 37° in 1 ml of Krebs-bicarbonate medium containing 1 mM L-[U-^{14}C] leucine (sp. act. 1 µCi/µmole), then rinsed briefly in ice-cold water and homogenized in 1 ml of 10% trichloroacetic acid. The homogenizer was rinsed twice each with 1 ml of 10% trichloroacetic acid; the combined homogenate and rinsings were heated for 5 min at 100°, then cooled in ice and filtered through a 2.1 cm Whatman GF/A glass fibre disc in a Millipore filter. The precipitate on the filter was washed three times each with 5 ml of ice-cold 10 mM L-leucine in 5% trichloroacetic acid, then three times each with 1 ml of absolute ethanol and then dried for 2 min at 110°. The filter discs were placed into counting vials with 1 ml of NCS tissue solubilizer (Amersham/Searle Corp) and the precipitated protein dissolved by heating for 2 h at 50°. Toluene scintillator (10 ml) was added and the radioactivity counted in a Philips model PW4510 liquid scintillation counter.

MATERIALS

Radioisotopes were obtained from the Radiochemical Centre, Amersham. Cycloheximide and puromycin were from Sigma Chemical Co., St. Louis, and chloramphenicol from Parke Davis Co. Ltd., Sydney. Beef insulin, twice re-

crystallized, was a gift from the Commonwealth Serum Laboratories, Melbourne. This was iodinated to a specific activity of 50-100 μCi/μg (0.14-0.28 atoms ^{125}I per mole insulin) and used at a concentration of 4 ng/ml (2).

RESULTS

Freshly isolated soleus muscles incorporated 79 nmoles leucine/g into protein over a 15-min incubation period (Fig. 1). When the muscles were preincubated at 37° before measuring protein synthesis, this was maintained for the first 30 min and then declined progressively; after 90 min the incorporation of leucine into protein had fallen to 54% of that measured in fresh muscle. Cycloheximide (0.2 mg/ml) added at the start of the preincubation period inhibited protein synthesis approximately 96%; the onset of this effect was very rapid. The inhibitory effect of puromycin (0.2 mg/ml) was somewhat slower in onset and reached a value of 93% after 90 min. The binding of ^{125}I-insulin by soleus muscle was not affected by preincubation in the presence of either inhibitor, nor was there any effect on muscle ATP (Table 1). Both cycloheximide and puromycin inhibited insulin-stimulated xylose uptake; puromycin, but not cycloheximide, also inhibited basal xylose uptake. These inhibitory effects were already evident when sugar transport was measured without prior exposure to the inhibitor, and became more pronounced after preincubation.

Part, if not all, of the residual leucine incorporation measured in the presence of cycloheximide will be due to mitochondrial protein synthesis (3). To see whether this played any role in the synthesis of insulin receptors, muscles were preincubated for 90 min in the presence of both cycloheximide and chloramphenicol. This combination should inhibit both cytoplasmic and mitochondrial protein synthesis in eukaryotes. Protein synthesis was inhibited 98% in the presence of cycloheximide (0.2 mg/ml) and chloramphenicol (0.1 mg/ml) (Fig. 1). At this concentration chloramphenicol did not affect the binding of ^{125}I-insulin, xylose uptake or muscle ATP (Table 2). When muscles were incubated with cycloheximide (0.2 mg/ml) and chloramphenicol (2 mg/ml), protein

TABLE 1 EFFECT OF CYCLOHEXIMIDE AND PUROMYCIN ON ^{125}I-INSULIN BINDING, XYLOSE UPTAKE AND ATP

Preincubation conditions			Xylose uptake (μmol/g per h)		
Time (min)	Addition	Bound ^{125}I-insulin (ng/g)	Basal	Insulin	ATP (μmol/g)
0	-	-	-	13.4 ± 0.4 (5)*	-
0	Cycloheximide	-	-	11.3 ± 0.4 (5)*	-
				P < 0.01	
90	-	0.47 ± 0.03 (6)	2.1 ± 0.3 (8)	10.9 ± 0.4 (6)	2.8 ± 0.1 (4)
90	Cycloheximide	0.43 ± 0.02 (6)	1.8 ± 0.2 (8)	8.5 ± 0.3 (6)	2.9 ± 0.1 (4)
				P < 0.005	
180	-	0.39 ± 0.02 (5)	2.3 ± 0.3 (5)	11.6 ± 0.3 (5)	2.0 ± 0.1 (5)
180	Cycloheximide	0.41 ± 0.03 (5)	2.2 ± 0.3 (5)	7.6 ± 1.2 (5)	2.2 ± 0.1 (5)
				P < 0.025	
0	-	-	3.8 ± 0.4 (10)	13.7 ± 0.4 (4)*	-
0	Puromycin	-	3.0 ± 0.4 (10)	11.3 ± 0.3 (4)*	-
			P < 0.01	*P < 0.05*	
90	-	0.47 ± 0.03 (6)	2.7 ± 0.1 (10)	11.1 ± 0.5 (5)	1.8 ± 0.2 (4)
90	Puromycin	0.53 ± 0.03 (6)	2.0 ± 0.1 (10)	6.2 ± 0.4 (5)	1.9 ± 0.1 (4)
			P < 0.01	*P < 0.005*	

^{125}I-insulin binding, xylose uptake and ATP were determined in soleus muscles after preincubation under aerobic conditions at 37° in the presence of cycloheximide (0.2 mg/ml), puromycin (0.2 mg/ml) and insulin (0.1 U/ml) as shown. Values are mean ± S.E.

* In these experiments the muscles were first incubated for 10 min in the presence of insulin before the inhibitors were added.

TABLE 2 EFFECT OF CYCLOHEXIMIDE AND CHLORAMPHENICOL ON ^{125}I-INSULIN BINDING, XYLOSE UPTAKE AND ATP

Preincubation conditions				Xylose uptake (μmol/g per h)		
Time (min)	Cyclo-heximide (mg/ml)	Chloram-phenicol (mg/ml)	Bound ^{125}I-insulin (ng/g)	Basal	Insulin	ATP (μmol/g)
90	0.2	-	0.53 ± 0.05 (5)	2.2 ± 0.1 (6)	8.5 ± 0.3 (4)	3.0 ± 0.2 (4)
90	0.2	0.1	0.53 ± 0.09 (5)	2.2 ± 0.1 (6)	9.1 ± 0.6 (4)	3.0 ± 0.1 (4)
90	0.2	-	0.50 ± 0.05 (6)	2.1 ± 0.1 (6)	7.8 ± 0.4 (5)	2.8 ± 0.2 (4)
90	0.2	2.0	0.16 ± 0.05 (6)	1.6 ± 0.1 (6)	1.7 ± 0.2 (5)	0.8 ± 0.1 (4)
			P < 0.005	*P < 0.01*	*P < 0.001*	*P < 0.001*
90	-	-	0.52 ± 0.09 (6)	2.4 ± 0.3 (7)	10.5 ± 1.0 (5)	2.6 ± 0.1 (4)
90	-	2.0	0.17 ± 0.05 (6)	1.1 ± 0.2 (7)	2.6 ± 0.2 (5)	0.8 ± 0.1 (4)
			P < 0.01	*P < 0.005*	*P < 0.005*	*P < 0.005*
0	-	-	-	3.3 ± 0.4 (9)	12.0 ± 0.8 (5)*	3.9 ± 0.2 (9)**
0	-	2.0	-	2.1 ± 0.2 (9)	7.1 ± 0.3 (5)*	3.8 ± 0.2 (9)**
				P < 0.005	*P < 0.01*	

^{125}I-insulin binding, xylose uptake and ATP were determined after preincubation under aerobic conditions at 37° in the presence of cycloheximide, chloramphenicol and insulin (0.1 U/ml) as shown. Values are mean ± S.E.

* In these experiments the muscles were first incubated for 10 min in the presence of insulin before the addition of chloramphenicol.

** Muscles incubated for 5 min ± chloramphenicol.

synthesis was inhibited by 99%. At this higher concentration chloramphenicol depressed ^{125}I-insulin binding by 68% and abolished the stimulatory effect of insulin on xylose uptake (Table 2); basal xylose uptake was also inhibited. From the ATP levels shown in Table 2 it is clear that this concentration of chloramphenicol had a marked effect on muscle energy metabolism. This is in agreement with the previous observation that, at this concentration, chloramphenicol inhibited respiration of rat liver and HeLa cells (4). Similar effects on ^{125}I-insulin binding, sugar transport and ATP were observed when the muscles were incubated in the presence of chloramphenicol (2 mg/ml) alone. The inhibitory effect of chloramphenicol (2 mg/ml) alone on muscle protein synthesis increased from a value of 25%, measured over the period 0-15 min, to a maximum of approximately 88% after 30 min exposure to the inhibitor (Fig. 1).

The inhibitory effects of chloramphenicol (2 mg/ml) on ^{125}I-insulin binding and insulin-stimulated xylose uptake could have been due to its lowering of muscle ATP levels, in terms of the ATP-dependence previously described (1, 2). However, if this were the only effect of chloramphenicol, then one would have expected the inhibitor to stimulate basal sugar transport, in accord with the well-documented action of anoxia and uncouplers (5). That it did not stimulate, but actually inhibited, basal xylose uptake suggested that, like puromycin, chloramphenicol may have some direct inhibitory effect on sugar transport. Chloramphenicol inhibited basal and insulin-stimulated xylose uptake when this was measured without prior exposure to the inhibitor (Table 2). There was no effect of chloramphenicol on muscle ATP levels during the course of these short-term incubations.

DISCUSSION

The primary aim of these studies was to determine whether the permissive effect of ATP on ^{125}I-insulin binding and on insulin-stimulated sugar transport by rat soleus muscle (1,2) was due to the lack of ATP for the synthesis of the insulin receptor. It is now clear that this is not so. Thus, there was no

effect on insulin binding when protein synthesis was inhibited over extended periods with cycloheximide, puromycin or a low concentration of chloramphenicol (0.1 mg/ml). The only effect on insulin binding was observed when muscles were pre-exposed to a toxic concentration of chloramphenicol (2 mg/ml). As this was found to disrupt energy metabolism, then it seems very likely that the inhibition of insulin binding observed under these conditions was due to the depletion of muscle ATP, consistent with the effects of anoxia and 2,4-dinitrophenol previously reported (2).

All of the inhibitors tested inhibited the process of sugar transport in soleus muscle. There was, however, a difference between the effect of cycloheximide, which only inhibited sugar transport in the presence of insulin, and of puromycin and chloramphenicol which inhibited basal sugar transport as well. There is relatively little information in the literature concerning the effect of protein synthesis inhibitors on muscle sugar transport. Hjalmarson (6) reported that puromycin inhibited basal xylose uptake by rat diaphragm in vitro; however, other workers found that there was no effect of this inhibitor on the uptake of glucose (7,8) or xylose (8,9). Buneaux et al. (10) found that cycloheximide did not affect the uptake of xylose by rat diaphragm incubated under basal conditions. The difference between the effects of cycloheximide and of puromycin and chloramphenicol (2 mg/ml) on basal sugar transport and the rapidity with which these inhibitory effects appeared, suggest that they are not due to the inhibition of protein synthesis, but rather to some effect more directly concerned with the process of sugar transport. The inhibitory effect of all three inhibitors tended to increase as the period of exposure was extended. This could have been due in part to the inhibition of protein synthesis; however, whether or not this is so has yet to be determined. Nevertheless, the effects on muscle sugar transport observed in the present studies, together with other effects on sugar transport in thymocytes (11) and glycogen metabolism in diaphragm (12), serve to emphasize that these inhibitors may have actions other than on protein synthesis alone.

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REFERENCES

1. Yu, K.T., and Gould, M.K. (1978) Amer. J. Physiol. 234, E407-E416.
2. Yu, K.T., and Gould, M.K. (1978) Amer. J. Physiol. (in press).
3. Schatz, G., and Mason, T.L. (1974) Annu. Rev. Biochem. 43, 51-87.
4. Firkin, F.C., and Linnane, A.W. (1968) Biochem. Biophys. Res. Commun. 32, 398-402.
5. Korbl, G.P., Sloan, I.G., and Gould, M.K. (1977) Biochim. Biophys. Acta 465, 93-109.
6. Hjalmarson, A. (1968) Acta Endocrinol. 57, Suppl. 126, 19-35.
7. Eboue-Bonis, D., Chambaut, A.M., Volfin, P., and Clauser, H. (1963) Nature 199, 1183-1184.
8. Søvik, O. (1965) Acta Physiol. Scand. 63, 325-335.
9. Carlin, H., and Hechter, O. (1964) Proc. Soc. Exp. Biol. Med. 115, 127-129.
10. Buneaux, J.J., Gounelle, J.C., and Raoul, Y. (1971) Horm. Metab. Res. 3, 94-97.
11. Yasmeen, D., Laird, A.J., Hume, D.A. and Weidemann, M.J. (1977) Biochim. Biophys. Acta 500, 89-102.
12. Søvik, O. (1966) Acta Physiol. Scand. 66, 307-315.