INSULIN-STIMULATED SUGAR TRANSPORT AND ^{125}I-INSULIN BINDING BY RAT SOLEUS MUSCLE: PERMISSIVE EFFECT OF ATP

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SUMMARY. The effect of insulin (0.1 U/ml) on the uptake of D-[U-^{14}C] xylose by rat soleus muscle was abolished by preincubation for 90 min under anaerobic conditions. This loss of insulin sensitivity was associated with decreased binding of ^{125}I-insulin. Prolonged anoxia also depleted muscle ATP. These results are in accord with the proposal that the action of insulin on sugar transport involves some ATP-dependent step. Aerobic incubation (90 min) with cycloheximide (200 μg/ml) did not affect ^{125}I-insulin binding. This suggests that the effect of prolonged anoxia was not due simply to any effect on the synthesis of the insulin receptor, but rather to some more direct effect of ATP on the interaction of insulin with its receptor.

The transport of sugars into the muscle cell is subject to regulation by external factors, notably insulin, and also by changes in the metabolic state of the muscle itself (1, 2). The mechanism through which the sugar transport system responds to changes in intracellular metabolism is imperfectly understood. Noting that transport was increased by a number of factors which tended to lower muscle ATP levels, Randle and Smith (3) proposed that ATP could act as a feedback inhibitor of sugar transport. Recent studies in this laboratory have suggested that ATP could have yet another role in the regulation of muscle sugar transport. Korbl et al. (4) observed that 2:4-dinitrophenol, which stimulated the uptake of D-xylose by soleus muscle under basal conditions, inhibited insulin-stimulated sugar transport. They also showed that insulin stimulated xylose uptake when freshly isolated muscles were incubated in the presence of 2:4-dinitrophenol, but had no effect after a period of preincubation with the uncoupler. This suggested that there may be some ATP-dependent process involved in the stimulatory effect of insulin. The experiments presented in this paper provide further evidence for such a permissive role for ATP.

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METHODS

Rat soleus muscles (approx. 30 mg from Wistar rats fed ad lib) were incubated for 30 or 90 min at 37° in 0.95 ml of Krebs-bicarbonate medium, pH 7.4, under an atmosphere of either 95% O_2-5% CO_2 or 95% N_2-5% CO_2. Following this preincubation, xylose uptake or muscle ATP was measured using methods previously described (4). The binding of ^{125}I-insulin was determined by a modification of the method of Wohltmann and Narahara (5): After preincubation at 37° under aerobic or anaerobic conditions, the muscles were blotted on filter paper dampened with Krebs-bicarbonate medium. One muscle from each pair was then incubated for 30 min at 25° under O_2-CO_2 in 1 ml of Krebs-bicarbonate medium containing 2 mg of bovine serum albumin and 4 ng of ^{125}I-insulin (25-50 nCi/ng); the other muscle was incubated under similar conditions in medium containing 4 ng of ^{125}I-insulin and 16 μg of unlabelled insulin. The muscles were rinsed briefly in ice-cold buffer, blotted on filter paper dampened with buffer, and then washed for 30 min at 0° in 5 ml of bicarbonate buffer containing 2 mg/ml of bovine serum albumin. The muscles were blotted and washed again, this time for 60 min at 0° in 5 ml of buffer-serum albumin, and then blotted on damp filter paper and frozen between aluminium plates chilled in dry ice. The frozen muscles were homogenized in 2 ml of 10% trichloroacetic acid. The homogenizer was rinsed twice with 2 ml of 10% trichloroacetic acid, and the combined homogenate and rinsings centrifuged for 15 min in a bench centrifuge. The precipitate was washed twice with 2 ml of 5% trichloroacetic acid, and then counted in a Philips PW4520 automatic gamma counter. The "specific" binding of ^{125}I-insulin was calculated as the difference between the ^{125}I-insulin which was bound in the absence ("total") and presence ("non-specific") of added cold insulin. Control experiments showed that non-specific ^{125}I-insulin binding was not further lowered when the concentration of added unlabelled insulin was increased 10-fold.

MATERIALS

D-[U-^{14}C]xylose and D-[1-^{3}H] sorbitol were obtained from the Radiochemical Centre, Amersham. Beef insulin, twice recrystallized, was a gift from the Commonwealth Serum Laboratories, Melbourne. For binding studies, this was iodinated using carrier-free Na[^{125}I] iodide from the Radiochemical

Centre, Amersham, according to the procedure described by Greenwood et al. (6). Crystalline enzymes used for the assay of ATP were from Boehringer Mannheim Australia Pty. Ltd., Melbourne. Cycloheximide was from Sigma Chemical Co., St. Louis.

RESULTS AND DISCUSSION

The experiments presented in Table 1 examined the effect of anaerobic preincubation on muscle ATP levels and on basal and insulin-stimulated rates of xylose uptake. As previously reported (4), the uptake of xylose by soleus muscle was markedly stimulated by insulin; this effect of insulin was still evident after preincubation for 90 min under aerobic conditions. Insulin also stimulated xylose uptake after anaerobic preincubation for 30 min. However, there was no effect of insulin after preincubation for 90 min under anaerobic conditions.

From the data presented in Table 2 it is clear that the lack of insulin responsiveness after prolonged anaerobic preincubation was associated with a reduced capacity to bind ^{125}I-insulin. The binding of ^{125}I-insulin was not modified by aerobic incubation for up to 90 min or by anaerobic preincubation for 30 min. Prolonged anaerobiosis depressed the "specific" binding of ^{125}I-insulin; there was no effect on the "non-specific" binding of ^{125}I-insulin, which remained essentially constant under all conditions. These effects of prolonged anoxia could be related to the ATP content of the muscles (Table 1). Thus, ATP levels were well maintained in aerobic muscles and in anaerobic muscles after 30 min. Conversely, ATP was essentially depleted after anaerobic preincubation for 90 min; in these muscles there was no effect of insulin on xylose uptake and ^{125}I-insulin binding was depressed. There was no obvious effect of insulin on muscle ATP levels under any of the experimental conditions employed.

It seems reasonable to assume that these effects on insulin-binding and insulin-stimulated sugar transport were due in some way to the lack of oxidative energy metabolism under prolonged anaerobic conditions. To determine whether this was due simply to the suppression of protein synthesis, the effect of cycloheximide on ^{125}I-insulin binding was examined. Control experiments

TABLE 1 EFFECT OF ANAEROBIC PREINCUBATION IN THE PRESENCE OR ABSENCE OF INSULIN ON XYLOSE UPTAKE AND MUSCLE ATP

The uptake of xylose (measured over 5 min at 37°) or muscle ATP was measured in soleus muscle pairs preincubated in Krebs-bicarbonate buffer ± insulin (0.1 U/ml) under O_2-CO_2 or N_2-CO_2 as shown. Values shown are mean ± S.E. (No.).

Pre-incubation (min)	Aerobic			Anaerobic		
	Basal	Insulin	P	Basal	Insulin	P
			Xylose Uptake (μmoles/g/h)			
30	1.7 ± 0.2 (4)	11.3 ± 0.8 (4)	<0.001	3.8 ± 0.5 (5)	8.9 ± 0.7 (5)	<0.02
90	1.5 ± 0.2 (5)	11.1 ± 0.4 (5)	<0.001	3.9 ± 0.3 (8)	4.2 ± 0.3 (8)	N.S.
			ATP (μmoles/g			
30	3.7 ± 0.2 (4)	3.6 ± 0.1 (4)	N.S.	2.7 ± 0.1 (4)	2.4 ± 0.3 (4)	N.S.
90	3.4 ± 0.2 (4)	3.2 ± 0.2 (4)	N.S.	0.1 ± 0.03 (4)	0.2 ± 0.1 (4)	N.S.

TABLE 2 EFFECT OF ANAEROBIC PREINCUBATION ON THE BINDING OF ^{125}I-INSULIN BY SOLEUS MUSCLE

Soleus muscle pairs were first preincubated at 37° in Krebs-bicarbonate buffer as shown and then incubated for 30 min at 25° in the presence of 4 ng/ml ^{125}I-insulin (25-50 nCi/ng) ± 16 μg/ml unlabelled insulin. Following the washing procedures detailed under "METHODS", the bound ^{125}I-insulin was counted. Values are mean ± S.E. of 5 observations.

	Preincubation			Bound ^{125}I-insulin (ng/g muscle)		
	Time (min)	Atmos	Cyclo-heximide (200 μg/ml)	Total	Non-specific	Specific
Expt. I	0	-	-	0.63 ± 0.04	0.23 ± 0.01	0.40 ± 0.04
	30	O_2-CO_2	-	0.64 ± 0.01	0.21 ± 0.02	0.43 ± 0.004
		N_2-CO_2	-	0.64 ± 0.03	0.23 ± 0.02	0.41 ± 0.02
	90	O_2-CO_2	-	0.77 ± 0.03	0.28 ± 0.02	0.49 ± 0.03
		N_2-CO_2	-	0.49 ± 0.03	0.30 ± 0.02	0.19 ± 0.03*
Expt. II	90	O_2-CO_2	-	0.87 ± 0.05	0.44 ± 0.03	0.43 ± 0.05
		O_2-CO_2	+	0.78 ± 0.05	0.35 ± 0.03	0.43 ± 0.05

* N_2-CO_2 *vs* O_2-CO_2, $P < 0.001$

(to be reported separately) showed that the incorporation of ^{14}C-labelled leucine into protein by soleus muscle was inhibited 95% by cycloheximide (200 μg/ml). This effect was very rapid and was evident within 10 min. When muscle protein synthesis was inhibited by preincubation with cycloheximide (200 μg/ml) for 90 min under O_2-CO_2, there was no effect on ^{125}I-insulin binding (Table 2). This suggests that the effect of anoxia on ^{125}I-insulin binding cannot be due to the inhibition of protein synthesis.

These experiments lend strong, although circumstantial, support to the proposal that there is an ATP-dependent step involved in the action of insulin on sugar transport and, furthermore, suggest that this is closely concerned with the initial hormone-receptor interaction. Whether or not this is so must await a more detailed examination of the effects described above. However, it would not be inappropriate at this point to consider a number of factors which could be involved in this system. The present results could be explained if the ability of the receptor to bind insulin were regulated by the phosphorylation of some membrane protein, possibly even the receptor itself. In this way, ATP could exert a modulatory influence on the binding of insulin and thus its biological action. At present, although this scheme is purely hypothetical, reports from a number of laboratories concerning the phosphorylation of muscle membrane proteins by cyclic AMP-dependent (7) and independent (8, 9) protein kinases lend credence at least to its feasibility.

One point which deserves further comment is why the biological effect of insulin on sugar transport was completely abolished, even though these muscles still retained an appreciable capacity to bind ^{125}I-insulin "specifically".* There are a number of possible explanations for this observation. The first relates to the heterogeneity of insulin receptors, which has been observed in studies on insulin-binding by muscle (10) and other tissues (11, 12). The present result could be explained if different receptors modulated the effects of insulin on sugar transport and on other insulin-sensitive systems. It is well established, for example, that the effects of insulin on sugar transport and on the activation of muscle glycogen synthase represent independent actions of the

* Other experiments showed that this residual binding of ^{125}I-insulin was not depressed further when the anaerobic incubation was extended from 90 to 120 min.

hormone (13, 14). This would imply that not all of these receptors were subject to regulation by ATP as proposed above.

The second explanation concerns the definition of the term "specific" binding itself. As introduced by Wohltmann and Narahara (5), "specific" denoted the association of insulin with a receptor such that it triggered some biological response. More recently the term has been used, in the study of insulin and other hormone receptors, to describe that portion of the bound labelled hormone which could be displaced by the addition of a large excess of unlabelled hormone. "Specific", in this sense, refers to the fact that the labelled insulin so bound could be displaced by cold insulin, or its biologically-active derivatives, but not by other hormones (10, 15). It is not clear, however, to what extent all of the displaceable insulin can be considered to be "specifically" bound according to the definition of Wohltmann and Narahara (5). The experiments of Rasio (16) showed that skeletal muscle could store ^{125}I-insulin in a biologically inert form and yet "specifically" as judged by its displacement by cold insulin. The release of insulin from exercising muscle (17) is a further indication of the presence of storage sites within the tissue. To the extent that these sites contribute to the measured "specific" binding, this could also explain the difference between ^{125}I-insulin binding and insulin stimulated transport reported above.

Finally, one must also consider that there may be some ATP-dependent step, subsequent to binding, concerned with the transmission of the insulin signal. In this regard, there are a number of ATP-driven ion pumps which could be involved in mediating the biological action of insulin. Effects of insulin on the Na^+/K^+-dependent ATPase (18, 19) and on the uptake of Mg^{2+} (20) have been reported. Similarly, current interest in the role of Ca^{2+} as a potential mediator of insulin action (1, 2) could indicate the involvement of a Ca^{2+}-dependent ATPase. Any or all of these systems could be responsible, along with depressed insulin-binding, for the suppression of the biological effect of insulin by prolonged anaerobiosis.

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REFERENCES

1. Elbrink, J., and Bihler, I. (1975) Science 188, 1177-1183.
2. Clausen, T. (1975) Curr. Top. Membrane Transp. 6, 169-226.
3. Randle, P.J., and Smith, G.H. (1958) Biochem. J. 70, 501-509.
4. Korbl, G.P., Sloan, I.G., and Gould, M.K. (1977) Biochim. Biophys. Acta 465, 93-109.
5. Wohltmann, H.J., and Narahara, H.T. (1966) J. Biol. Chem. 241, 4931-4949.
6. Greenwood, F.C., Hunter, W.M., and Glover, J.S. (1963) Biochem. J. 89, 114-123.
7. Sulakhe, P.V., and Drummond, G.I. (1974) Arch. Biochem. Biophys. 161, 448-455.
8. Pinkett, M.O., and Perlman, R.L. (1974) Biochim. Biophys. Acta 372, 379-387.
9. Andrew, C.G., Almon, R.R., and Appel, S.H. (1975) J. Biol. Chem. 250, 3972-3980.
10. Olefsky, J., Bacon, V.C., and Baur, S. (1976) Metabolism 25, 179-191.
11. Hammond, J.M., Jarett, L., Mariz, I.K., and Daughaday, W.H. (1972) Biochem. Biophys. Res. Commun. 49, 1122-1128.
12. Kahn, R., Freychet, P., Neville, D.M., and Roth, J. (1972) Diabetes 21, 334-335.
13. Sovik, O. (1965) Acta Physiol. Scand. 63, 325-335.
14. Eboue-Bonis, D., Chambaut, A.M., Volfin, P. and Clauser, H. (1967) Bull. Soc. Chim. Biol. 49, 415-431.
15. Freychet, P., Roth, J., and Neville, D.M. (1971) Proc. Natl. Acad. Sci. 68, 1833-1837.
16. Rasio, E.A. (1969) Diabetologia 5, 416-419.
17. Dieterle, P., Gmeiner, K.-H., Henner, J., Dieterle, C., and Schwarz, K. (1972) Horm. Metab. Res. 4, 54.
18. Brodal, B.P., Jebens, E., Oy, V., and Iversen, O.-J. (1974) Nature 249, 41-43.
19. Gavryck, W.A., Moore, R.D., and Thompson, R.C. (1975) J. Physiol. Lond. 252, 43-58.
20. Lostroh, A.J., and Krahl, M.E. (1973) Biochim. Biophys. Acta 291, 260-268.