Control by insulin of protein synthesis in muscle

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The concentrations of the free amino acids in the intracellular water of skeletal muscle are higher than their equivalent concentrations in plasma. These concentration gradients can be maintained during short-term incubations in vitro. Moreover, both in vitro and in vivo, skeletal muscle will accumulate some radioactive amino acids against a concentration gradient. Since some of this radioactivity may be incorporated into protein, it might be thought that the intracellular amino acids formed the immediate precursor pool for protein synthesis. This simple view has been questioned by several workers (Kipnis, Reiss & Helmreich, 1961; Gaal, Beney & Szekely, 1965). Indeed, Hider, Fern & London (1969), using a double incubation technique with both ^{14}C- and ^{3}H-labelled amino acids, demonstrated in vitro that amino acid from the medium was incorporated direct into protein in preference to that from the tissue. This surprising result was confirmed by the same group (Hider, Fern & London, 1971*a*) when they suggested that a tissue pool, distinct from the intracellular pool and termed 'the extracellular pool', contained the amino acids to be incorporated direct into protein. This pool, which approximated to the extracellular space, contained amino acids at a concentration midway between that present in the incubation medium and that in the intracellular fluid.

In an isolated muscle preparation it is easy to think of a diffusion barrier between medium and tissue. The muscle is removed from its natural environment where each muscle cell, or bundle of muscle fibres, is in close proximity to a capillary network that permeates the interstices of the tissue. In the in vitro incubation, the medium must diffuse through the interstitial spaces to reach the tissue, a process that is unlikely to be instantaneous. A method to overcome this objection would involve an in vivo perfusion system of skeletal muscle such as that recently developed for other purposes (Ruderman, Houghton & Hems, 1971) or the utilization of a Langendorff preparation for perfusing the isolated heart. With this latter technique, Morgan, Earl, Broadus, Wolpert, Giger & Jefferson (1971) investigated the role of the intracellular and extracellular pools in the synthesis of protein. These authors concluded that the rates of incorporation of lysine and glycine could be explained more on the basis of an intracellular than an extracellular precursor pool. However, the results of these experiments differed in several ways from those obtained with the extensor digitorum preparation (Hider *et al.* 1971*a*). In the studies of Morgan *et al.* (1971) there was no detectable loss of free intracellular glycine or lysine into the protein pool or into the extracellular fluid. Indeed, as the radioactivity was washed out of the extracellular fluid, the intracellular fluid:extracellular fluid distribution ratio approached infinity.

Another method for the study of protein metabolism is the isolated cell obtained either direct from the animal or grown in tissue culture (see review by Munro, 1969). In systems such as these, where evidence for compartmentation cannot be explained on the basis of anatomical artefacts, there appears to be a source other than that of the total free intracellular pool that is utilized for protein synthesis. The most recent evidence for this concept is derived from work with HeLa cells (Righetti, Little & Wolf, 1971) where the specific activity of leucine in newly induced ferritin was 30% lower than that of a pre-equilibrated radioactive free intracellular pool. Comparable results with phenylalanine gave a figure of 62%. These authors concluded that part of the amino acids in protein came from the reutilization of amino acids derived from protein breakdown that had not equilibrated with the free pool in the cells. The importance of degraded protein as a source of amino acids has been pointed out by Gan & Jeffay (1967) who showed that in fasting rats 65% of the free amino acid pool of muscle was derived from protein and that the figure dropped to 30% in the fed state.

Although experimental evidence had been adduced to show that the total free intracellular pool of amino acids was not incorporated direct into the protein of skeletal muscle in vitro, the mechanism by which insulin stimulated protein synthesis was unclear. Many groups of workers have indicated that insulin could stimulate intracellular accumulation of some, but by no means all, amino acids by muscle (see review by Manchester, 1970), and herein lay a possible explanation for the way in which the hormone stimulated the synthesis of protein, namely by increasing the intracellular supply of amino acids. Such a hypothesis had the attraction of explaining the effects of insulin on protein synthesis on the basis of its known action on membrane transport. The apparent paradox whereby insulin stimulated the intracellular accumulation of only a few of the amino acids found in protein was resolved by the observation that when protein synthesis was inhibited by puromycin an insulin effect on the cellular uptake of more amino acids was unmasked (Wool, 1965).

Recent work on the manner in which insulin stimulated protein synthesis has concentrated on the control of initiation (Wool, Stirewalt, Kurihara, Low, Bailey & Oyer, 1968) rather than on a link with membrane transport. However, since there is strong evidence that insulin has a primary action at the cell membrane rather than within the cell (Cuatrecasas 1969), it seemed likely that the initial effect of the hormone would be found in some function that took place at the membrane.

With this thought in mind we have investigated the effect of the hormone on the amino acid pools of the extensor digitorum longus muscle of the rat (Hider, Fern & London, 1971b). From our earlier studies (Hider et al. 1971a) we had defined this in terms of a four-compartment model where an extracellular fluid pool was interposed between the in vitro incubation medium and the intracellular pools of amino acids in protein or distributed through the intracellular water.

Insulin enhanced protein synthesis in this preparation by appreciably shortening the delay that was observed before a linear rate of amino acid incorporation had been established. Further experimentation revealed that this effect on protein synthesis

was not due to the hormone having increased the radioactivity of the intracellular pool; no insulin effect was apparent when the intracellular pool had been preloaded with radioactive amino acid. Only when insulin and the label were simultaneously present in the medium did the hormone stimulate incorporation.

This experiment made two points. First, that the hormone did not make the intracellular amino acids directly available for incorporation, and second, as a corollary, that earlier observations (Hider *et al.* 1969) on the compartmentation of amino acids held true for the insulin-stimulated state. This aspect of the problem had previously been investigated by Wool & Krahl (1964) who incubated muscle in the presence of [^{14}C]pyruvate and [^{12}C]alanine. They showed that ^{14}C-radioactivity was incorporated into protein and concluded that, since the ^{14}C was incorporated into alanine within the tissue, the source of amino acid entering protein was the intracellular pool. They based this conclusion on the assumption that any radioactive amino acid effluxed would be diluted to insignificance by the ^{12}C-labelled amino acid in the medium. The falsity of this assumption has recently been revealed by Hider & Meade (1972); they have shown that the effect of this 'cold' alanine on the extracellular specific radioactivity of [^{14}C]alanine formed from pyruvate was very much less than imagined largely because increasing the alanine concentration in the medium markedly increases the amount of radioactive alanine effluxed into the medium after its formation from pyruvate. Indeed in their studies, Hider & Meade (1972) found that the amount of radioactivity associated with alanine increased to a much greater extent in the medium than in the tissue when the alanine concentration in the medium was raised.

Several other features emerged from this study that could shed light on the way in which insulin stimulated protein synthesis. Apart from confirming the observations of others (Manchester & Young, 1960; Wool, 1965) on the effects of insulin on the intracellular pools of glycine and leucine, it was demonstrated (Hider *et al.* 1971*b*) that the hormone consistently lowered the specific radioactivity of these two amino acids in the extracellular pool. These last results could be explained in two ways: either, the bidirectional flux of amino acids was being stimulated with an increased efflux from a relatively unlabelled intracellular pool into the more highly labelled extracellular pool; or the extracellular pool could itself be compartmentalized with the highly labelled compartment selectively incorporated into protein. These possibilities are not mutually exclusive and have not been distinguished one from another by the experiments conducted thus far. However, if the precursor pool is more closely identified with the extracellular rather than the intracellular space the effect of insulin on incorporation of radioactive label could not be explained on the basis of the hormone increasing the specific radioactivity of the pool; for it was the opposite pattern which was observed. If, as has already been suggested, the extracellular fluid pool is itself compartmentalized and the immediate precursor pool closely associated with the cell membrane (Hendler, 1962), it could be that insulin facilitated the binding to tRNA (Davey & Manchester, 1969) from a specific pool near to the membrane. This would link the lowering of the specific radioactivity found in extracellular fluid amino acids (Hider *et al.* 1971*b*) to the rise in the specific

radioactivity of aminoacyl-tRNA derivatives following exposure to the hormone (Davey & Manchester, 1969).

Since insulin could stimulate both the bidirectional flux of amino acids across the cell membrane as well as amino acid incorporation into protein, it seemed necessary to carry out further studies to elucidate this interrelationship. It has been previously shown by others (Manchester, 1966) that insulin would have an effect on protein synthesis when no effect on intracellular accumulation of amino acid could be demonstrated. This phenomenon was observed when Na^+ was excluded from the incubation medium. We have recently found (Barnes, Boswell and London, unpublished observations) in similar experiments with the extensor digitorum longus muscle that insulin can stimulate protein synthesis in muscle incubated in sodium-free medium but that this effect is much smaller than the effect seen in the presence of sodium. When ouabain, an inhibitor of Na^+- and K^+-dependent ATPase and hence of sodium transport, is present in the medium, a stimulation of protein synthesis is seen to an intermediate degree. Measurement of the tissue pools of glycine showed that both ouabain and absence of sodium depressed the hormonal stimulation of amino acid accumulation; indeed, muscles incubated in sodium-free medium did not respond at all with an increase in glycine uptake. In these, as in earlier studies, there was a decrease in glycine specific radioactivity in those muscles incubated with insulin in Krebs–Ringer bicarbonate buffer. However, when ouabain was presented in the medium or when sodium was excluded, insulin did not produce any change in specific radioactivity of the extracellular glycine. Thus, the pattern of changes in these studies was that manoeuvres which inhibited the bidirectional fluxes of amino acids across the cell membrane also reduced the response of incorporation to insulin. This phenomenon has been previously commented on (Goldstein & Reddy, 1970).

Now, since we have already shown that insulin does not stimulate protein synthesis by way of its effect on the inward transport of amino acids, we must postulate that, if changes in the membrane are causally related to changes in protein synthesis, it is through some other membrane effect. The observation that insulin reduces the insulin space, but not the mannitol space (Creese & Northover, 1961; Fritz & Knobil, 1963), might be interpreted as showing an increase in the size of an extracellular compartment through which small molecules can permeate at the expense of another open to both large and small molecules. If the small molecule compartment contained the amino acid precursor pool for protein synthesis and insulin enhanced the entry of amino acid from the medium into this pool, a membrane phenomenon linked to the effect of the hormone on protein synthesis would have been demonstrated. Some such effect might resemble the recent demonstration in adipocytes that insulin regulates the assembly of microtubules (Soifer, Braun & Hechter, 1971). The link still missing from the chain is how these changes could bear on the initiation of protein synthesis in the ribosome.

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