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Review Articles

A Partial View of the Mechanism of Insulin Action

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Insulin was discovered 60 years ago [1]; since then its structure has been determined by the pioneer work of Sanger [2] and by Hodgkin, Blundell and colleagues [3]. Moreover the principle effects of insulin on carbohydrate, fat and protein metabolism have been well recognised, at least in general terms, for some 20 years [4–7], yet, we do not have a satisfactory description at the molecular level of how this hormone brings about its wide range of effects on target cells. This is certainly not due to any lack of attention to the problem by research workers. The literature on this subject is vast and, in the absence of any generally acceptable hypothesis, it also tends to be rather confused and certainly conflicting. Since it is quite unrealistic to survey the whole field, we make no apology for concentrating on certain aspects in this article.

Insulin has both long and short term effects on the metabolism of its target cells. The long term effects involve changes in both general and specific protein synthesis and breakdown, while short term effects are those brought about solely through changes in the activity of pre-existing enzymes and membrane transporters. To a large extent, we will neglect all aspects of long term regulation including protein synthesis and amino acid metabolism. In fact, advances in these areas have been delayed by the lack of satisfactory preparations suitable for probing the mechanisms involved in the specific induction and repression of enzymes by insulin in vitro. This situation will probably change rapidly as a number of such preparations have been reported recently. Particularly promising are the 3T3-L1 preadipocyte cell line [8, 9] and also the maintenance liver cell cultures from young rats in which glucokinase activity has been shown to be rapidly induced on exposure to insulin in the presence of glucose [10].

In the rest of this review we will concentrate on those studies concerned with short term effects of insulin on carbohydrate and fat metabolism in the cells of liver, muscle and, particularly, adipose tissue. All these cells have specific insulin receptors on the outward face of the plasma membrane but we will not be considering the nature of these receptors nor their interaction with insulin nor the mechanisms which may govern the number of insulin receptors. These aspects have all been well covered in other recent reviews [11–13]. We shall address ourselves to the problem of the process or processes whereby alterations in the level of occupancy of the plasma membrane insulin-receptors lead to a wide range of intracellular changes. The assumption will be made that neither the whole insulin molecule nor part of it *needs* to enter the cells of its target tissue to bring about the effects of insulin. There seems little doubt that insulin bound to its receptor can be "internalized" into liver and probably other cells [14, 15], however, the process appears to be too slow to represent the means whereby insulin could bring about its short term effects. On the other hand, antibodies to the insulin receptor appear to initiate all the short term intracellular effects of insulin itself [12, 16–21]. This supports the view, but perhaps not entirely conclusively, that the mechanism of action of insulin does not require the entry of any part of the insulin molecule. It would appear that the interaction of either insulin or the anti-insulin receptor antibodies with the insulin receptors on the cell membrane brings about changes in the receptors, perhaps including aggregation [11, 12], which initiate the sequence of events leading to the observed intracellular effects.

The short term effects of insulin on rates of fat and carbohydrate metabolism can be easily summarised [see also 4–7, 22]. The hormone stimulates glucose uptake into muscle and fat cells; in muscle, much of the increased glucose taken up is converted to glycogen while in fat cells it is mainly converted to the glycerol

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Table 1. Stable changes in enzyme and carrier activity following short term exposure of tissues to insulin

Enzyme or carrier	Tissue	Change in activity	Possible mechanism	References
Glucose uptake				
Glucose transport	W, (SM, H)	increase (V*)	? translocation	26–30
Cyclic AMP metabolism				
Phosphodiesterase (low K_m)	W, L	increase (V*)	? (W); phosphorylation (L)	34–44
Protein kinase (Cyclic AMP dep.)	W, SM	decrease (V^+ in W; Ka* in SM)	association of R and C subunits	45–48
Glycogen metabolism				
Glycogen synthase	H, SM, W, L	increase (K_a*)	dephosphorylation	49–54, 86
Phosphorylase kinase	L	decrease (V^+)	dephosphorylation	51, 55
Phosphorylase	W, L	decrease ($K_a{}^+$)	dephosphorylation	52, 53, 55–57, 86
Glycolysis and pyruvate oxidation				
Pyruvate kinase	L, W	increase ($K_s{}^+$ in L; K_s* in W)	dephosphorylation (L); ? (W)	58–62
Pyruvate dehydrogenase	W, B, L (MG)	increase (V*)	dephosphorylation	63–71
Lipid metabolism				
Acetyl CoA carboxylase	W, B, L	increase (V*)	polymerisation and phosphorylation	71–82
Hydroxymethyl glutaryl CoA reductase	L	increase (V*)	dephosphorylation	83, 84
Triacylglycerol lipase	W	decrease (V^+)	dephosphorylation	85–86

Column 2 and elsewhere: *W*, white fat; *B*, brown fat; *H*, heart muscle; *SM*, skeletal muscle; *L*, liver; *MG*, mammary gland. Column 3: *V*, change in V_{max}; K_s, change in K_m for substrate; K_a change in sensitivity to appropriate activator; * change in activity most marked in absence of other hormones; $^+$, change in activity most marked under conditions of increased cell cyclic AMP concentrations

and fatty acid moieties of triacylglycerol. In fat cells, insulin also inhibits lipolysis especially when this has been stimulated by other hormones. In addition, insulin has a range of important effects on liver cell metabolism. These include the inhibition of glucose release and gluconeogenesis on the one hand and the promotion of glycogen storage and triacylglycerol synthesis on the other. We start this review by considering the key enzymes and membrane carriers which exhibit changes in activity following exposure of target cells to insulin. It seems reasonable to assume that if the molecular basis for these changes in activity is established then important clues to the mechanism of insulin action should emerge.

Sites of Insulin Action on Carbohydrate and Fat Metabolism

The first site of insulin action to be recognised was the transfer of glucose across the plasma membrane of muscle and fat cells [23–25]. Insulin causes an increase in the apparent maximum activity (V_{max}) of the glucose carrier with little or no change in the apparent affinity (K_m) of the carrier for glucose [see 26]. Despite many attempts it has not proved possible to demonstrate this effect by addition of insulin to broken cell preparations [26], although prior exposure of fat cells to insulin has been found to lead to an increase in the glucose transport activity of membrane vesicles prepared subsequently [27]. The molecular basis of the effect of insulin is still a matter of speculation but important advances towards an understanding have been made by Czech and his colleagues. They have shown that the carrier may be solubilised with appropriate detergents, partially purified and then inserted in an active form into phospholipid vesicles [26, 28, 29]. Moreover, the effect of insulin appears to persist through these manipulations [29, 30] indicating that insulin may cause some covalent modification, for example a change in disulphide bonds [22] or phosphorylation of the glucose carrier. An alternative explanation, for which there is mounting evidence, is that insulin causes the recruitment of carrier molecules from intracellular sites to the plasma membrane [31–33].

The change in glucose carrier activity is by no means the only stable change that persists into extracts of cells previously exposed to insulin. A wide range of enzymes important in carbohydrate and fat metabolism also show this phenomenon and well-established examples are listed in Table 1. All these changes in activity appear to be essentially independent of the effect of insulin on glucose transport since they can be observed in the absence of extracellular glucose. However, in a number of cases including glycogen synthase and acetyl CoA carboxylase, the effects of insulin may be enhanced in the presence of glucose [50, 73]. In addition to the enzymes listed in

Table 1, insulin may also cause the activation of liver phosphofructokinase [87–90] and a number of the enzymes involved in the esterification pathway of triacylglycerol synthesis in fat and liver cells [91–98].

Table 1 gives an indication of the nature of the change in activity. It should be noted that the changes in kinetic behaviour may involve changes in maximum activity (V_{max}) or changes in apparent affinity for substrate (K_s) or for an activator (K_a). Table 1 also shows the possible mechanisms although as we will be emphasising later direct evidence is often lacking. Since the changes in activity all persist into tissue extracts it is to be expected that many of the effects will be brought about by covalent modification. Indeed, in many instances the changes in kinetic behaviour of the enzymes which persist in extracts of intact cells previously exposed to insulin are indistinguishable from those which have been found to occur with alterations in the extent of phosphorylation of the isolated enzymes. However, covalent modification is not the only possible explanation for changes in enzyme activity, since they may also be brought about by alterations in the amount of a tightly-binding effector associated with the enzyme or by association or dissociation of enzyme subunits. Alterations in the activity of liver cell phosphofructokinase with glucagon and maybe insulin would appear to be examples of the former possibility since the changes in activity probably involve changes in the amount of an activator (fructose 2,6-bisphosphate) bound to the enzyme [87–90]. In contrast, changes in the activity of cyclic AMP dependent protein kinase observed in fat and other cells with adrenaline, insulin and other hormones are probably caused by the dissociation of this enzyme into separate receptor and catalytic subunits which under appropriate conditions persist into tissue extracts [47]. Nevertheless, it is important to emphasise that usually alterations in the activity of an enzyme in an intact cell which are the result of changes in the concentration of substrate or effector acting directly on the enzyme will not be apparent when the enzyme is subsequently assayed in a tissue extract.

Taken together the changes in enzyme activity observed after exposure to insulin (Table 1) offer a reasonable explanation of many of the principle changes in carbohydrate and fat metabolism in cells. Thus the reciprocal changes in the activity of glycogen synthase and phosphorylase are presumably the basis of the promotion of glycogen synthesis in a number of tissues. The increased rate of fatty acid synthesis in white fat cells appears to involve the parallel activation of pyruvate dehydrogenase, acetyl CoA carboxylase and pyruvate kinase [99] and probably this will also prove to be the case for liver, brown fat and mammary tissue cells. Inhibition of fat cell lipolysis by insulin clearly must involve inhibition of triacylglycerol lipase but the exact site or sites of insulin action in the stimulation of esterification is far less evident. Saggerson and his colleagues have observed that exposure of fat cells to adrenaline leads to decreases in the activities of fatty acyl CoA synthetase, glycerol phosphate acyltransferase, diacylglycerol acyltransferase and phosphatidate phosphohydrolase; these decreases are diminished in the presence of insulin [91–98]. It is possible that the changes in glycerol phosphate acyltransferase are brought about by changes in phosphorylation of the enzyme since Nimmo [100, 101] has shown this enzyme to be reversibly inhibited following treatment with cyclic AMP, ATP and protein kinase. However, it would also appear possible that some of the changes in activity reported by Saggerson's group may be caused by the accumulation of fatty acids and fatty acyl CoA esters in the cells incubated with adrenaline [98].

A number of the effects of insulin on liver and fat cells are most apparent in the presence of hormones which increase the concentration of cyclic AMP in the target cells. Examples are the changes in the activities of phosphorylase, phosphorylase kinase, liver pyruvate kinase and fat cell triacylglycerol lipase. These correlate with observations that the inhibition of glycogen breakdown, gluconeogenesis and triacylglycerol breakdown by insulin are, for the most part, only evident in the presence of another hormone which increases cyclic AMP such as glucagon (liver) or adrenaline (fat cells). Since all these enzymes have the potential for regulation either directly or indirectly by cyclic AMP dependent protein kinase, it would appear likely that the decreases in tissue cyclic AMP which have often been found under these conditions may partially explain the action of insulin in these cases. However, even in these cases changes in cyclic AMP do not seem to offer an entirely adequate explanation (see below).

Dephosphorylation of these and other enzymes may also involve an increase in the activity of phosphoprotein phosphatase. One way this might be brought about is through a decrease in the inhibitory activity of "inhibitor-1" [102–104]. This is a small protein (subunit Mr about 25,000) which when phosphorylated on a specific threonine by cyclic AMP dependent protein kinase inhibits the general phosphoprotein phosphatase in many mammalian cells. Some evidence for a small diminution in the ability of this peptide to inhibit phosphoprotein phosphatase activity has been obtained following exposure of muscle to insulin [105, 106] but other studies [107, 108] were unable to detect any effect of the hormone.

From the point of view of establishing the mechanism of action of insulin, it seems reasonable to con-

Table 2. *Effects of insulin on specific protein phosphorylation in rat fat cells*

Band no.[a]	Approximate subunit M_r ($\times 10^{-3}$)	Cell location	Identity	Changes in overall phosphorylation		Comments	References
				with insulin	with adrenaline		
1	230	Cytoplasm	Acetyl CoA carboxylase	increase (~ 10%)	increase (~ 50%)	Phosphorylated at multiple sites	80, 116, 117 125, 126
2	130	Cytoplasm	ATP-citrate lyase	increase (2–3fold)	increase (1.5-two-fold)	Enzyme activity unchanged	18, 110–124
	85	Cytoplasm/fat droplet	Triacylglycerol lipase		increase (two-fold: reversed by insulin)	Minor peak	127–128
4A	65	Cytoplasm	?	no effect?	increase (> fourfold; reversed by insulin)		111, 113, 115, 117, 118, 120
5	61	Plasma membrane	?	increase (~ 30%)	increase (~ 60%)		129
6	42	Mitochondria	Pyruvate dehydrogenase (α subunit)	decrease (~ 30%)	variable effects	Phosphorylated at multiple sites	67, 114
7	35	Ribosomes?	S_6?	increase (2–3fold)	increase (1.5-twofold)	Comparable changes also observed in 3T3-L1 cultures	18, 67, 114, 130, 131
9	22	Cytoplasm	Related to inhibitor-1	increase (> 4fold)	increase (2–threefold)	Minor peak; soluble in 2% trichloroacetic acid	18, 132

[a] Band number refers to the numbers allocated in this laboratory to the main bands of [^{32}P] labelled phosphoproteins in a whole cell extract which are separated by SDS-polyacrylamide gel electrophoresis [18, 67, 114, 116, 117, 129, 132]

In addition to the above, Benjamin and colleagues have reported that insulin also results in the increased phosphorylation of a protein (subunit Mr 50–62,000) associated with the 'fat cake' [111, 118]

centrate on those effects of insulin which are apparent under basal conditions (i. e. in the absence of other hormones). Under these conditions decreases in the tissue level of cyclic AMP are very small and usually undetectable [7, 22, 86, 109]. The best examples are the activation of glycogen synthase in muscle and pyruvate dehydrogenase and acetyl CoA carboxylase in fat cells and some emphasis will be given in the remainder of this article to these enzymes. Other examples in this group are the low K_m form of cyclic nucleotide phosphodiesterase and hydroxymethylglutaryl CoA reductase but the mechanisms regulating these enzymes are not so well understood at present, mainly because these enzymes are membrane bound and thus difficult to study.

It is evident from Table 1 that many of the effects of insulin appear to be consistent with changes in the state of phosphorylation of specific enzymes – in most cases with dephosphorylation. However, it is obviously important that the proposed changes in phosphorylation are shown actually to occur in the cells exposed to insulin. As will become apparent in the next section this has not been directly demonstrated for the majority of the enzymes given in Table 1.

Effects of Insulin on Specific Protein Phosphorylation in Intact Cells

The effects of hormones on the level of phosphorylation of specific proteins within intact cells can be explored by incubating the cells with medium containing [^{32}P]-phosphate and then separating the labelled phosphoproteins by a variety of techniques including sodium dodecyl sulphate-polyacrylamide gel (SDS) electrophoresis. Benjamin and Singer [110, 111] were the first to apply such techniques to the investigation of the effects of insulin on protein phosphorylation in rat fat cells. Since then this approach has been further developed by others including ourselves [112–115]. In particular, subcellular fractionation and specific immunoprecipitation have been employed to separate and identify a number of the major labelled phosphoproteins [18, 67, 112–117, 129].

Table 2 lists the principle alterations in protein phosphorylation which have been reported in fat cells exposed to insulin or adrenaline. From Table 1, it might have been expected that the common characteristic of insulin action would be the dephosphorylation of a number of key intracellular proteins. In fact, under basal conditions, the dephosphorylation of only one protein has been demonstrated, namely of the α-subunit of the pyruvate dehydrogenase complex [67] which confirms the view that the activation of this mitochondrial enzyme in fat cells exposed to insulin results from a greater proportion of this enzyme being

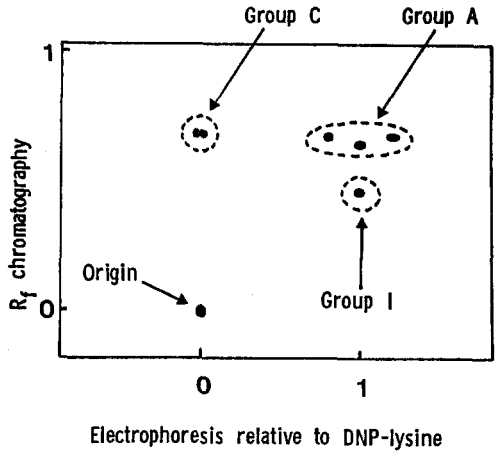
present in its active non-phosphorylated form [67–71]. The most obvious effect of insulin is to cause an increase in the phosphorylation of at least five different proteins. Before discussing these phosphoproteins in more detail, it is necessary to be confident that the observed increases in the incorporation of [^{32}P]-phosphate into these intracellular phosphoproteins do indeed represent increases in the overall level of phosphorylation of the individual proteins. It is possible, in principle, that insulin might stimulate phosphoprotein phosphatase activity and thus lead to the observed increase in [^{32}P] incorporation simply because the turnover of certain phosphate groups would be increased. In such circumstances, increases in [^{32}P] incorporation could occur without any increase in the actual number of phosphate groups at the sites concerned. To ensure that this situation does not occur, differences in steady-state levels of incorporation of [^{32}P] must be determined. Steady-state labelling of the intracellular phosphoproteins is reached after about 60 min for isolated fat cells, then hormones are added and incubations continued until a new pattern of steady-state labelling is achieved (less than 10 min for both insulin and adrenaline). Confirmation that changes in turnover do not contribute to the observed alterations in [^{32}P] incorporation requires that the hormone effects are reversible upon subsequent removal of the hormone. Recently it has been demonstrated that the two fold increases in phosphorylation of the proteins of subunit (M_r) 130,000 (band 2) and 22,000 (band 9) observed in small pieces of white adipose tissue exposed to insulin are essentially reversed on subsequently adding anti-insulin serum to the incubation medium (G. Belsham unpublished observations).

The increased phosphorylation of a protein of subunit M_r of 130,000 was the first effect of insulin on specific protein phosphorylation in fat cells to be recognised [110–113] mainly because this protein is present in particularly high concentrations in fat cells. More recently, immunoprecipitation has revealed that the protein is ATP-citrate lyase [122–124]. Increased phosphorylation of this protein is also observed in cells exposed to adrenaline. The physiological role of these changes in phosphorylation remain a mystery as changes in phosphorylation have not been found to have any effect on the catalytic activity of ATP-citrate lyase [122–124, 133]. Studies in our laboratory have shown that insulin also causes very substantial increases in the overall phosphorylation of two other smaller intracellular proteins with subunit M_r of 35,000 and 22,000 [18, 67, 114, 132]. The identity of these proteins has yet to be completely established.

The protein of M_r 35,000 is associated with the "microsomal" fraction and may be the ribosomal protein S_6. Insulin has been reported to increase the phosphorylation of this protein in cultures of 3T3-L1 cells [130, 131] but, maybe, decrease its phosphorylation in the liver [134]. The protein of M_r 22,000 may be of particular importance in insulin action. It has a number of unusual properties in common with inhibitor-1 of the general phosphoprotein phosphatase mentioned in the previous section. For example, it is not precipitated by 2% trichloracetic acid or by boiling. It also has approximately the same subunit M_r as determined by SDS-polyacrylamide gel electrophoresis [132]. As expected, increased phosphorylation of the protein is evident after exposure of the cells to adrenaline which increases cyclic AMP concentration [132]. The even greater degree of phosphorylation of this protein in cells exposed to insulin does not fit into current thinking about the role of inhibitor-1 [105, 106, 135]. Either the labelled protein in fat cells is not inhibitor-1 but rather a closely related protein or present concepts concerning the regulatory role of inhibitor-1 are incomplete.

Detection of changes in the phosphorylation of a phosphoprotein (subunit M_r 85,000) which is most probably triacylglycerol lipase has been achieved by Belfrage and colleagues [127, 128]. Due to the high specific activity of this enzyme, its concentration in fat cells is very low compared with many other phosphoproteins.

So far we have only considered changes in overall incorporation of [^{32}P] into specific intracellular proteins. Many proteins may be phosphorylated at more than one site and thus changes in overall phosphorylation may give only limited information. Pyruvate dehydrogenase and acetyl CoA carboxylase are both good examples; studies with purified preparations of these two enzymes have shown that they may be phosphorylated at multiple sites [81, 136–139]. In the case of pyruvate dehydrogenase, phosphorylation at three separate sites on the α-subunits (by the tightly bound kinase) has been demonstrated. Phosphorylation of only one of the three sites is required to achieve full inactivation of the enzyme [136]. Randle and his colleagues have argued on the basis of studies on the enzyme from heart muscle that the phosphorylation of the other two sites may serve as a means of regulating the activity of pyruvate dehydrogenase phosphatase [140–143]. Thus the possibility arises that the effect of insulin on fat cell pyruvate dehydrogenase activity could involve changes in the relative occupancy of the three sites. Techniques were therefore developed involving rapid immuno-isolation, trypsin digestion and separation of phosphopeptides which allow the relative level of phosphorylation of the sites to be assessed [67]. All three sites were shown to be phosphorylated in intact fat cells but no evidence for any change in the relative occupancy of the three sites was



Treatment of fat cells	Incorporation of ^{32}P into phosphopeptides of group (as % control ★) C	A	I
No hormone	100★	92±27	10±4
Insulin	98±9	116±24	51±9
Adrenaline	108±16	228±54	26±8
Insulin plus adrenaline	118±26	274±61	63±14

Fig. 1. Effects of insulin and adrenaline on the phosphorylation of specific sites on acetyl CoA carboxylase in rat epididymal fat cells. Fat cells were incubated with [^{32}P] phosphate and hormones as appropriate and [^{32}P]-acetyl CoA carboxylase isolated by immunoprecipitation [116, 117]. Following trypsin digestion the [^{32}P]-phosphopeptides were separated by two-dimensional analysis on thin layer cellulose plates [139]. Dinitrophenyl-lysine (DNP-lysine) was used as an internal reference for mobility in the electrophoresis dimension. In the chromatography dimension mobility is expressed as a fraction *(Rf)* of that of the solvent front. (DNP-lysine moved with an Rf of 0.8–0.9 in the chromatography dimension.) The [^{32}P]-phosphopeptides were located by autoradiography, assigned to groups C, A or I according to observed mobility as indicated in the figure, and then quantified by densitometry. Results are expressed as mean ± SEM (ten observations)

found following treatment with insulin and it was concluded that insulin decreases the level of phosphorylation of all three sites in parallel [67]. Similar techniques have now been applied to acetyl CoA carboxylase with markedly differing results. As indicated in Figure 1, tryptic digestion of acetyl CoA carboxylase from fat cells gives rise to a number of different labelled phosphopeptides. After exposure of the cells to adrenaline, the activity of acetyl CoA carboxylase is diminished and this is associated with increased [^{32}P]-labelling of certain phosphopeptides. These appear to match those obtained with purified rabbit mammary gland enzyme phosphorylated with cyclic AMP dependent protein kinase [139]. In contrast, after exposure to insulin (which increases the activity of acetyl CoA carboxylase), a marked increase in phosphorylation of a single different peptide was apparent and no change was discernible in the phosphopeptides principally affected by adrenaline. We conclude that inhibition of acetyl CoA carboxylase by adrenaline is probably brought about by the increased phosphorylation of specific sites on the enzyme by the cyclic-AMP dependent protein kinase. Activation by insulin also appears to be associated with *increased* phosphorylation but at a different site and presumably brought about by a different protein kinase. It seems reasonable to extend this line of thinking to the other phosphoproteins in fat cells which exhibit increased phosphorylation in the presence of both insulin and adrenaline (ATP-citrate lyase and proteins of subunit M_r of 61,000, 35,000 and 22,000). This has lead us to search for a cyclic AMP independent protein kinase which might play a central role in insulin action and this will be discussed further below.

Very few studies on the effects of insulin on specific protein phosphorylation have been carried out using cells other than isolated rat epididymal fat cells. In liver cells under basal conditions, it has been reported that insulin causes the increased phosphorylation of an unidentified cytoplasmic protein of subunit M_r 46,000 [144] as well as ATP-citrate lyase (M_r 130,000) [145]. We know of no studies using insulin in muscle preparations although they are clearly technically possible [146–147]. Studies into the phosphorylation of glycogen synthase within muscle cells may be especially illuminating. Extensive studies on the purified muscle enzyme have shown that it is possible to phosphorylate a total of six to nine different sites on the protein [148–151]. In general terms, it has been found that as more sites are phosphorylated, the enzyme becomes increasingly insensitive to activation by glucose 6-phosphate. Phosphorylation at specific sites on the purified muscle enzyme has been shown to be brought about by a number of different protein kinases including cyclic AMP dependent protein kinase and phosphorylase b-kinase [152] plus other less well characterised kinases [148–151] including a Ca^{2+} activated protein kinase which can be distinguished from phosphorylase b-kinase [153]. Clearly studies concerned with establishing the mechanism of action of insulin on glycogen synthase would be more soundly based if the actual changes in phosphorylation of the various sites of the enzyme in muscle cells following exposure to insulin could be determined. It is even possible that insulin may lead to an increase in the

phosphorylation of a specific site or sites on glycogen synthase as found for acetyl CoA carboxylase in fat cells.

Consideration of Some Hypotheses of Insulin Action

Changes in Cyclic Nucleotides

It is well established that under appropriate conditions, insulin can cause a diminution in the concentration of cyclic AMP and a transient increase in the concentration of cyclic GMP in fat and liver cells. However, it is quite evident that these changes cannot offer a complete or satisfactory explanation for the mechanism of action of insulin. Indeed, in muscle cells no changes in cyclic nucleotide concentrations have been found [7, 108, 154–156]. This field has been reviewed extensively by others [7, 22, 86, 109, 157–159] and only an outline will be given here.

Under basal conditions (i. e. in the absence of other hormones), little or no effect of insulin on cyclic AMP levels in fat or liver cells is found, although many effects of insulin are manifested under these conditions (Tables 1 and 2). Convincing decreases in cyclic AMP concentrations can be observed when these cells are incubated with other hormones which increase cyclic AMP. As mentioned earlier, these decreases in cyclic AMP may offer a partial explanation of the ability of insulin to reverse the effects of adrenaline on lipolysis in fat cells and the effects of glucagon on gluconeogenesis and glycogen breakdown in liver cells. However, the decreases, especially in fat cells, have often been shown to be insufficient to provide a complete explanation [86, 160, 161] but not always [162].

No role has been found for the transient rise in cyclic GMP observed in fat and liver cells exposed to insulin [22, 157]. Other agents such as carbachol and nor-adrenaline can bring about similar changes without having the characteristic effects of insulin [163, 164]. In contrast, the rise in cyclic GMP with insulin is greatly diminished in fat cells incubated in Ca^{2+} free medium but the metabolic effects of insulin remain essentially unaltered [163].

Although changes in the cyclic nucleotides might only play a rather minor role in bringing about the effects of insulin, it is still important to establish the mechanisms underlying the changes since this may help to identify other perhaps more fundamental elements in insulin action. To date, most of the emphasis has been focussed on the means whereby insulin decreases the concentration of cyclic AMP.

There have been reports of the inhibition of adenyl cyclase activity in plasma membrane preparations by insulin [165–168], but others have been unable to demonstrate such effects [7]. In contrast, it has been found in many laboratories that exposure of fat or liver cells to insulin leads to an increase in the activity of a low K_m particulate phosphodiesterase [34–44] (Table 1). However, the role of this activation is somewhat mysterious as conditions which result in high levels of cyclic AMP in liver and fat cells also lead to activation of phosphodiesterase [36, 38–40]. In fact, it would appear that under the very conditions where insulin causes the most convincing diminution in levels of cyclic AMP, there is little or no effect of insulin on this phosphodiesterase.

Marchmont and Houslay have recently reported evidence showing that the activity of phosphodiesterase associated with liver plasma membranes is doubled upon incubation of the membranes with insulin in the presence of ATP and cyclic AMP [43]. It appears that this doubling of activity is brought about by a cyclic AMP-dependent protein kinase embedded in the liver plasma membrane and regulated by insulin binding to its receptor [42–44]. The physiological importance of these very interesting findings remain to be established since the effects of insulin on the activity and phosphorylation of this phosphodiesterase activity have yet to be demonstrated in intact liver cells. Moreover, activation of a cyclic AMP-dependent protein kinase does not seem to offer a satisfactory explanation for other intracellular effects of insulin.

Changes in the Concentration of Calcium Ions (Ca^{2+})

It has been suggested that an increase in cytoplasmic Ca^{2+} may be a key element in the mechanism of action of insulin [169, 170]. The principle evidence for this hypothesis has been obtained from the study of ^{45}Ca efflux from preloaded fat cells, although the actual observations have been rather contradictory. Thus, Clausen and Martin [171] reported that insulin increases ^{45}Ca efflux while Kissebah and co-workers found that insulin inhibited efflux especially in the presence of adrenaline [172, 173]. Amazingly, the two groups both came to the same conclusion, namely that insulin increases cytoplasmic Ca^{2+}. Since, as mentioned above, extracellular Ca^{2+} does not appear to be required for insulin action, it has to be assumed that any rise in cytoplasmic Ca^{2+} must be brought about by the release of Ca^{2+} from intracellular sites, perhaps on the plasma membrane. Further evidence that insulin may alter cell Ca^{2+} has been presented by Pershadsingh and McDonald who have found inhibition of Ca^{2+}-dependent ATPase activity in fat cell membranes on addition of insulin [174] or from insulin-treated cells [175]. However, the effects of insulin in these studies were rather modest and were only ob-

served with fat cell membranes at a very low magnesium concentration.

One attraction of this hypothesis is that a rise in cytoplasmic Ca^{2+} would be expected to result in an increase in intramitochondrial Ca^{2+} [176] which could explain the activation of pyruvate dehydrogenase since pyruvate dehydrogenase phosphate phosphatase is activated by Ca^{2+} [177]. The effect of insulin on pyruvate dehydrogenase activity persists during the preparation and subsequent incubation of mitochondria from fat cells [63, 64, 178, 179]. This phenomenon has allowed evidence to be obtained which strongly suggests that the effect of insulin does involve stimulation of the phosphatase (rather than inhibition of the kinase) [178].

The weakness of the hypothesis is the lack of evidence that a rise in cytoplasmic Ca^{2+} could bring about any of the cytoplasmic effects of insulin. In contrast, such a rise would be expected to bring about a range of effects which are certainly not characteristic of insulin, including an increase in glycogen breakdown because of the activation of phosphorylase kinase. Indeed, in the rat liver, insulin opposes the effects of phenylephrine on glycogen breakdown and gluconeogenesis-effects which are widely considered to be brought about by an increase in cytoplasmic Ca^{2+} [180–182]. In any case, it is possible to envisage other mechanisms whereby insulin may increase the intramitochondrial level of Ca^{2+} and thus perhaps influence pyruvate dehydrogenase activity without the need for an increase in cytoplasmic Ca^{2+}. For example, exposure of cells to insulin could result in a change in the activity of one of the transport systems which determine the distribution of Ca^{2+} across the inner mitochondrial membrane [183, 184]. Jarett and his colleagues have suggested that insulin may alter the relationship between bound and free forms of Ca^{2+} associated with fat cell mitochondria [185].

Role of Hydrogen Peroxide (H_2O_2)

It has been proposed that H_2O_2 may act as a second messenger for insulin. Modest concentrations of H_2O_2 bring about a range of effects on fat cell metabolism which are rather similar to those seen with insulin, including increases in the conversion of glucose to glyceride glycerol and fatty acids and inhibition of lipolysis [186, 187]. Insulin also increases the oxidation of formate in intact fat cells. This has been taken as indicating that insulin can initiate the formation of H_2O_2 [188] although as far as we are aware there is no evidence that the oxidation of formate necessarily involves H_2O_2. More direct evidence for the view that insulin may stimulate the formation of H_2O_2 has been given by Muckerjee and Lynn [189]. They have reported that exposure of a crude fat cell membrane preparation to insulin results in the stimulation of a NADPH oxidase which forms H_2O_2 and that increased release of H_2O_2 could be detected in intact fat cells incubated with insulin [189]. However, these key observations have yet to be confirmed by others [see 187, 188].

Another difficulty with the view that H_2O_2 acts in any sense as a second messenger for insulin is that it is far from obvious how an increase in cell H_2O_2 could initiate any of the range of insulin effects. It has been reported that exposure of intact mitochondria to low concentrations of peroxides can result in the activation of pyruvate dehydrogenase [190] but we have been unable to obtain any activation which could not be accounted for simply by a decrease in the mitochondrial ADP/ATP ratio [J. McCormack, W. A. Hughes and R. M. Denton, unpublished observations].

Role of a Peptide Mediator

Larner and colleagues have extracted hind limb muscle from control and insulin-treated rats with acetic acid. After boiling and removal of denatured protein, the components in the extract were then separated by paper and sephadex G-25 chromatography. Evidence was obtained that one fraction contained a substance, probably a small peptide of 10–20 amino acids, which not only inhibited cyclic AMP dependent protein kinase but also activated glycogen synthase phosphate phosphatase. The amount or activity of this substance was apparently greater in fractions derived from insulin treated tissue [154, 191]. These findings led Larner and his group to suggest that "this peptide or peptide-like substance may constitute an insulin mediator" and that it might be released in some way from the plasma membrane following interaction of insulin with the insulin receptors [191]. If insulin caused the intracellular release of such a mediator it might explain the activation of glycogen synthase in muscle which is assumed to involve dephosphorylation but occurs in the absence of any detectable change in the concentration of cyclic AMP [7, 108, 154–156]. Many aspects of this interesting hypothesis remain to be established. Most importantly, the "peptide" needs to be purified and characterised. Significantly, further purification using paper electrophoresis appears to have revealed the presence of two mutually antagonistic agents in the fractions used in the original studies [192]. There also may be a problem of stoichiometry in this hypothesis which will be discussed in the final section.

Seals, Jarett and colleagues have reported that they are able to demonstrate an effect of insulin on fat

cell pyruvate dehydrogenase in a cell free system comprising of fat cell plasma membranes and mitochondria [193–200]. They have concluded that insulin causes the release of a factor from the plasma membranes, similar if not identical to that described by Larner's group, which is then capable of activating pyruvate dehydrogenase phosphate phosphatase and thus increasing the activity of pyruvate dehydrogenase in the mitochondria. These are potentially exciting findings but it is at present difficult to assess their physiological importance for a number of reasons including those outlined below.

The treatment of the mitochondrial fraction (including freezing) probably resulted in the pyruvate dehydrogenase system no longer being entirely enclosed within an intact inner-mitochondrial membrane and thus might allow interactions which could not occur in vivo. The actual changes in activity observed were small compared with those found in vivo, and also varied greatly between studies (compare [195, 196] and [199]). Moreover the changes in activity may not necessarily reflect alterations in phosphorylation since estimates of the proportion of the total complex in its active form were not routinely made in these studies. Finally most of the studies were carried out at 50 μmol/l calcium chloride and 50 μmol/l magnesium chloride so the phosphatase was probably exposed to saturating concentrations of Ca^{2+} but sub-optimal concentrations of magnesium: this is the reverse of the likely situation in vivo.

Despite much effort, we have not found it possible to obtain any appreciable effect of insulin on pyruvate dehydrogenase activity in systems similar to those used in the studies of Seals and Jarett in this laboratory (W. A. Hughes, R. M. Denton, J. G. McCormack, unpublished observations).

Activation of a Cyclic Nucleotide Independent Protein Kinase

We have already emphasised that exposure of fat cells to insulin leads to an increase in phosphorylation of at least five different intracellular proteins (Table 2). The possibility must therefore be entertained that a fundamental aspect of the mechanism of action of insulin might be activation of a protein kinase presumably quite distinct from the better known Ca^{2+} and cyclic nucleotide sensitive protein kinases.

The first evidence suggesting that such a kinase might exist was presented by Benjamin and Singer [111] who examined the ATP-dependent phosphorylation of proteins in a high-speed supernatant fraction of adipose tissue previously incubated in the presence or absence of insulin. They found that the extent of phosphorylation of a protein of subunit M_r 140,000 (now known to be ATP-citrate lyase) was increased in fractions from insulin-treated tissue. A similar type of approach has recently been employed to investigate the phosphorylation of the ribosomal protein S_6 in 3T3-L1 cell extracts. Again it was observed that phosphorylation was enhanced in extracts derived from insulin treated cells [131]. In both instances, the cyclic AMP independent kinases involved have been partly characterised and they may phosphorylate these proteins at different sites from those phosphorylated by cyclic AMP dependent protein kinase [201, 202]. Unfortunately, the physiological importance of the phosphorylation of both ATP-citrate lyase and the ribosomal protein S_6 remain obscure as no changes in activity or function have been observed with alterations in phosphorylation. Nevertheless, this pattern of phosphorylation fits in very well with our own recent studies on acetyl CoA carboxylase in fat cells described in an earlier section.

It will be recalled that the phosphorylation of acetyl CoA carboxylase in fat cells appears to be increased following exposure of cells either to insulin or to adrenaline. However, different sites are phosphorylated and appear to cause the observed increase and decrease in activity respectively. The sites phosphorylated in cells exposed to adrenaline appear to be the same as those phosphorylated by cyclic AMP dependent protein kinase [139]. We have searched for a separate protein kinase which might be involved in the action of insulin on this enzyme and have recently found such an activity – apparently associated with the plasma membrane. As indicated in Figure 2, incubation of acetyl CoA carboxylase with purified fat cell plasma membranes and ATP resulted in a marked increase in activity and this increase was associated with phosphorylation of the enzyme. The protein kinase appeared to be insensitive to cyclic nucleotides and Ca^{2+} [80].

With the above findings as background we have formulated the simple working hypothesis indicated in Figure 3. The essential feature is that the binding of insulin to its plasma membrane receptors leads to the activation of a protein kinase associated with the plasma membrane without the need for the generation of an intracellular second messenger or mediator such as a cyclic nucleotide, metal ion or small peptide. The activated kinase would then bring about the increased phosphorylation of a range of intracellular proteins and enzymes involved directly or indirectly in bringing about the observed effects of insulin. The simplest means of activation we can envisage is that the kinase dissociates from specific sites on the inner face of the plasma membrane following the formation of insulin-receptor complexes. This form of activation is rather analogous to the dissociation of the catalytic subunits

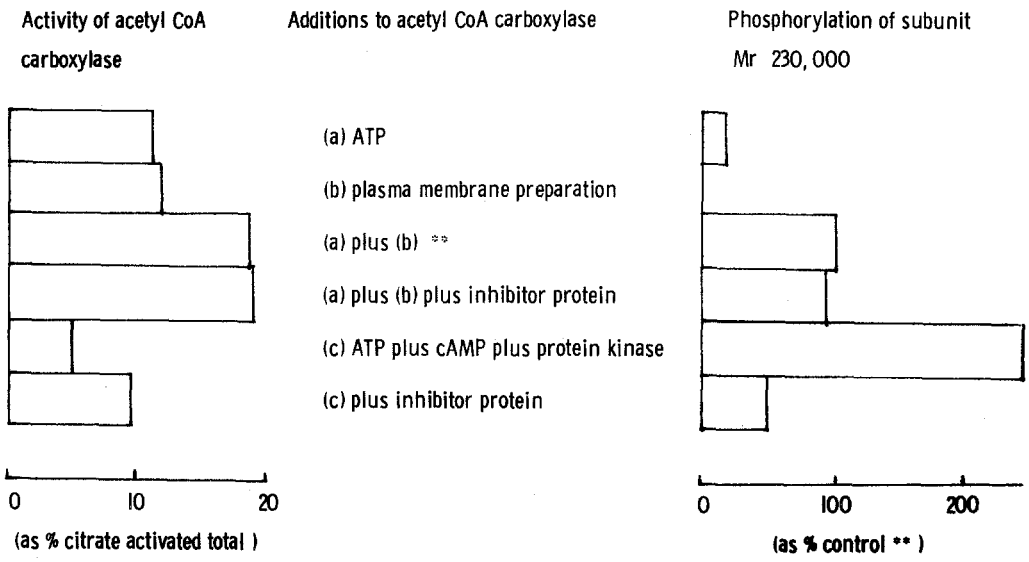


Fig. 2. Activation of fat cell acetyl CoA carboxylase following phosphorylation by a plasma membrane-associated cyclic AMP independent kinase. Partially purified acetyl CoA carboxylase was incubated with the additions indicated. To determine phosphorylation, [γ-^{32}P]-ATP was used and incorporation into acetyl CoA carboxylase measured following sodium dodecyl-sulphate-polyacrylamide gel electrophoresis. Fat cell plasma membranes were prepared as described by Belsham et al. [129]. Results taken from Brownsey et al. [80] where further details are given including the preparations of cyclic AMP dependent protein kinase and its inhibitor protein used. ** indicates control condition

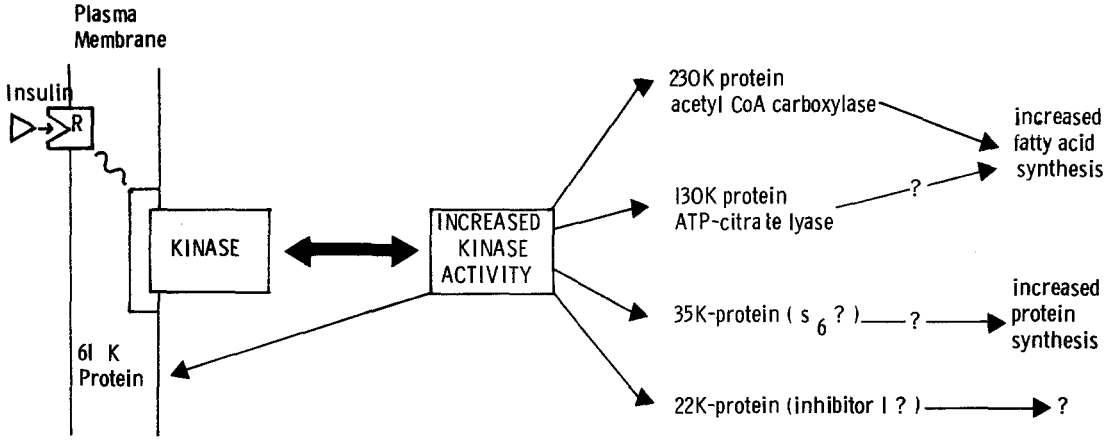


Fig. 3. Working hypothesis for the mechanism of action of insulin in fat cells

of the cyclic AMP dependent protein kinase initiated by the binding of cyclic AMP to the regulatory subunits. Such a mechanism would explain why persistant increases in the ability of high-speed supernatants to phosphorylate ATP-citrate lyase and the ribosomal protein S_6 have been observed following the exposure of cells to insulin.

Epidermal growth factor may act in a similar fashion to that envisaged in Figure 3 for insulin. The binding of this growth factor to specific receptors on the outside of A-431 epidermoid carcinoma cells has been shown to activate a membrane-bound protein kinase. This kinase appears to phosphorylate itself plus a range of soluble proteins [203–205].

Several studies have been carried out in which the effects of the direct addition of insulin on the activity of protein kinases in plasma membrane preparations have been investigated. Unfortunately, the results, are rather confusing which no doubt reflects the inherent difficulties in this type of study. Seals et al. report a decreased labelling of a fat cell plasma membrane protein of subunit M_r 120,000 (in addition to the changes in the labelling of pyruvate dehydrogenase discussed above) [193, 194]. Tran and Desbuquois

found a small diminution in the overall phosphorylation of proteins associated with rat liver plasma membranes apparently largely due to the decreased phosphorylation of two proteins of subunit M_r 110,000 and 60,000 [206]. However, Marchmont and Houslay have reported that incubation of rat liver plasma membranes with insulin in the presence of cyclic AMP causes the decreased phosphorylation of two integral proteins of subunit M_r 140,000 and 80,000 while increasing the phosphorylation of three peripheral membrane proteins (subunit M_r 52,000, 28,000 and 14,000) [42]. Most compatible with the hypothesis under discussion are the studies of Walaas and colleagues [207, 208]. These studies indicated that a plasma membrane fraction prepared from rat hind-limb muscle contained protein kinase activity which was activated on addition of insulin especially in the presence of GTP (guanosne 5'-phosphate). The activated kinase phosphorylated both an endogenous substrate M_r 16,000) as well as added histone II a. In none of the above cases have the observed changes in phosphorylation been shown to occur within intact cells incubated with insulin [129].

Final Comments

It can be estimated that the number of plasma membrane insulin receptors which need to be occupied to initiate a maximum response is in the range of 10^7–10^9 for each µl of cytoplasm of liver, muscle and fat cells [11, 209]. If it is assumed that only one molecule of a mediator or messenger of insulin action is generated and released into the cytoplasm for each occupied insulin receptor, the cytoplasmic concentration of the mediator would only be in the range 0.01 to 1 nmol/l. The concentrations of likely targets such as cyclic AMP dependent protein kinase, the general phosphoprotein phosphatase, pyruvate dehydrogenase phosphatase, phosphorylase kinase, inhibitor-1 are far greater than this as all are probably present at more than 0.1 µmol/l. Therefore hundreds, if not thousands of copies of the mediator must be released per occupied insulin receptor or some other form of amplification is required. The release from the inside of the plasma membrane of a protein kinase able to phosphorylate many intracellular protein molecules clearly meets this requirement for amplification, whereas the direct release of a peptide mediator as envisaged by Larner [154, 191, 192] would not.

A major weakness with our working hypothesis (illustrated in Figure 3) is that it offers no immediate explanation for those effects of insulin which appear to involve the dephosphorylation of certain enzymes including glycogen synthase, phosphorylase kinase, triglyceride lipase, and hydroxymethyl glutaryl CoA reductase in the cytoplasm and pyruvate dehydrogenase within mitochondria. To date, dephosphorylation following exposure of cells to insulin has only been demonstrated directly in intact cells for fat cell triacylglycerol lipase (in the presence of noradrenaline) [127, 128] and for fat cell pyruvate dehydrogenase [67]. We would like to suggest that the phosphorylation of the protein of subunit M_r 22,000 observed in insulin treated fat cells may turn out to play an important role in initiating the dephosphorylation of some cytoplasmic enzymes. The protein has very similar physical properties to inhibitor-1 which upon phosphorylation by cyclic AMP dependent protein kinase inhibits the activity of the "general" phosphoprotein phosphatase. It does not seem unreasonable to suppose that the phosphorylation of a related protein or even the same protein on different sites might have exactly the opposite effect and activate this phosphatase.

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