Pyruvate Dehydrogenase Activation in Adipocyte Mitochondria by an Insulin-Generated Mediator from Muscle

Abstract. *Material in a chromatographic fraction from an extract of insulin-treated muscle stimulated pyruvate dehydrogenase activity in adipocyte mitochondria. This action was similar to insulin's activation of the enzyme in a plasma membrane-mitochondria mixture. Neither the chromatographic fraction nor insulin required adenosine triphosphate or magnesium ion (Mg^{2+}), suggesting that both agents acted through a calcium-sensitive phosphatase. This fraction may contain a chemical mediator of insulin action.*

Insulin rapidly initiates a series of biological responses in the adipocyte after the hormone interacts with its receptor on the plasma membrane. Some form of a second messenger or chemical mediator system must be involved in generating these biological responses, but this messenger or mediator has yet to be identified. We have described a subcellular system consisting of plasma membranes and mitochondria from rat adipocytes on which insulin has direct effects (*1-4*). The initial observation was that addition of insulin to a highly enriched preparation of plasma membranes altered the labeling of the membrane by γ^{32}P-labeled adenosine triphosphate (ATP) (*1*). Two phosphoproteins were insulin-sensitive, a plasma membrane component of 120,000 daltons and a 42,000-dalton component of mitochondria that contaminated the plasma membrane preparation (*2*). The 42,000-dalton phosphoprotein was the alpha subunit of pyruvate dehydrogenase, and its insulin sensitivity in the subcellular system required the presence of plasma membranes (*3*). These studies suggested that insulin's interaction with the plasma membrane generated some chemical mediator or second messenger which decreased the phosphorylation of the mitochondrial enzyme and that under these conditions insulin would also stimulate pyruvate dehydrogenase activity. This action would be consistent with the mechanism proposed for insulin's activation of this enzyme in the intact adipocyte (*5*). Subsequent studies showed that insulin specifically activated pyruvate dehydrogenase activity in this subcellular system, but only in the presence of plasma membranes (*4*). The next major step was to identify the proposed second messenger in this system.

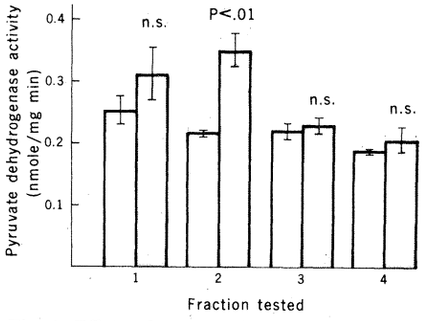

Fig. 1. Effect of extracts from control and insulin-treated muscle on pyruvate dehydrogenase activity of adipocyte mitochondria. Lyophilized samples of each fraction from the muscle extracts separated chromatographically on Sephadex G-25 (*6*) were dissolved to their original volume of 20 μl in 0.05*N* formic acid, and 10 μl was added to a 200-μl mixture containing 100 μg of adipocyte mitochondria (*4*), 50 μM Mg^{2+}, 50 μM Ca^{2+}, and 250 μM ATP in 50 m*M* K_2HPO_4 buffer, *p*H 6.5 at 37°C. After incubation for 5 minutes, pyruvate dehydrogenase activity was measured by the addition of 0.25 m*M* [1-^{14}C]pyruvate, 0.1 m*M* NAD (nicotinamide adenine dinucleotide), 0.1 m*M* coenzyme A, 0.1 m*M* cocarboxylase, and 1.0 m*M* dithiothreitol. The assay was carried out for 2 minutes at *p*H 6.5, and enzyme activity was determined as the amount of $^{14}CO_2$ produced (*4*). The respective fractions obtained from chromatographic separation of control and insulin-treated muscle are compared. The statistical significance of the difference between experimental and control samples was determined by the *t*-test.

Larner *et al.* (*6*) have described a factor generated by muscle in the presence of insulin, which appears to be a potential cellular mediator of insulin action. We have tested the ability of this substance to activate pyruvate dehydrogenase activity in the adipocyte mitochondrial preparation and compared its effects on the enzyme to those caused by insulin in the plasma membrane-mitochondria mixture.

The first four fractions from the chromatographic separation (Sephadex G-25 column) of extracts from insulin-treated muscle (*6*) were tested for their ability to stimulate pyruvate dehydrogenase activity of isolated mitochondria compared to their respective fractions from column chromatographic separation of control muscle extract. Prior to testing we did not know which fraction contained the substance identified (*6*) as the chemical mediator which inhibited the activity of the cyclic adenosine monophosphate (AMP)-dependent glycogen synthase kinase. Data were combined from two experiments in which fraction 2 was the only fraction from the insulin-treated muscle extract which significantly stimulated pyruvate dehydrogenase activity (Fig. 1). Larner *et al.* (*6*) have also shown that this fraction contains the greatest concentration of the active substance. Fraction 1 produced a small but not statistically significant increase in pyruvate dehydrogenase activity while fractions 3 and 4 had no effect.

Table I compares the ability of insulin to stimulate pyruvate dehydrogenase activity in a plasma membrane-mitochondria mixture to the ability of fraction 2 to stimulate the enzyme activity when added directly to isolated mitochondria. Initial experiments were performed in the presence of ATP, which suppresses pyruvate dehydrogenase activity to its lowest level (*1*). Under this condition both fraction 2 and insulin similarly stimulated the enzyme activity in their respective incubation conditions, the former by 22 percent and the latter by 32 percent. This stimulation is similar in magnitude to that reported for insulin, concanavalin A, and antibody to insulin receptor in the plasma membrane-mitochondria mixture (*4*).

The omission of ATP from the incubation medium resulted in higher basal activity (*4*). Both insulin and fraction 2 stimulated the enzyme activity in the absence of ATP by a greater absolute amount but by a slightly lesser percent-

Table 1. Stimulation of pyruvate dehydrogenase by fraction 2 and insulin in the presence and absence of ATP. Lyophilized samples of fraction 2 from control and insulin-treated muscle (*6*) were dissolved to their original volume of 10 μl in 0.05*N* formic acid and added to mitochondria, in which pyruvate dehydrogenase activity was then measured as described in the legend of Fig. 1. Enzyme activity was compared in the presence and absence of 250 μM ATP. The absolute and percentage difference between samples from control and insulin-treated muscle are presented. Insulin (100 μU/ml) or bovine serum albumin as a control (0.0005 percent) was added to a mixture containing 40 μg of adipocyte plasma membranes and 100 μg of mitochondria, 50 μM Mg^{2+}, 50 μM Ca^{2+}, in 50 m*M* K_2HPO_4, *p*H 7.4 at 37°C, with or without 250 μM ATP. The samples were incubated 5 minutes and pyruvate dehydrogenase activity was measured as described in the legend of Fig. 1, but at *p*H 7.4. The absolute and percentage differences between control and insulin samples is shown and is the mean of two studies.

Experimental condition	Absolute change (nmole/mg-min)	Percentage change
Fraction 2		
+ ATP	0.541	21.9
− ATP	1.107	17.6
Insulin		
+ ATP	0.422	31.8
− ATP	0.878	16.1

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age than in the presence of ATP. In a separate set of measurements, neither fraction 2 nor insulin required the 50 μM Mg^{2+} normally present in the incubation to produce a stimulation of pyruvate dehydrogenase activity (data not shown).

The above results demonstrate that the factor isolated from insulin-treated muscle, which has been suggested by Larner *et al.* (*6*) to be the chemical mediator of insulin action, mimics insulin's activation of pyruvate dehydrogenase activity in a subcellular preparation from rat adipocytes. We identified the chromatographic fraction containing the insulin-generated factor from muscle as the one with the greatest activity reported by Larner *et al.* (*6*). This substance activated pyruvate dehydrogenase activity under incubation conditions similar to those under which insulin decreased phosphorylation of the alpha subunit of pyruvate dehydrogenase (*2*, *3*) and activated the enzyme (*4*). Both insulin and fraction 2 increased pyruvate dehydrogenase activity in the presence of Mg^{2+}, Ca^{2+}, and ATP and in the absence of ATP or Mg^{2+} and ATP. The effects of insulin on pyruvate dehydrogenase activity in the adipocyte subcellular system occurred only in the presence of both plasma membranes and mitochondria, whereas fraction 2 acted directly on mitochondria in the absence of plasma membranes. This suggests that insulin in this subcellular system generates a chemical mediator by its interaction with the plasma membrane and that fraction 2 may contain this substance.

The mechanism by which insulin and fraction 2, as well as concanavalin A and antibody to insulin receptor (*4*), stimulate pyruvate dehydrogenase activity appears to involve activation of a phosphatase activity and not inhibition of a kinase activity. This conclusion is based on the ability of these agents to act when no ATP is included in the incubation medium. Since the mitochondria themselves contain no measurable ATP (data not shown), these agents cannot be acting by inhibiting protein kinase activity. This is consistent with observations on the intact adipocyte which suggest that insulin stimulates pyruvate dehydrogenase by activating a calcium-sensitive phosphatase which dephosphorylates the alpha subunit of the enzyme leading to an increase in the active dephosphorylated form of the enzyme (*5*). This concept is supported by the observations of McDonald *et al.* (*7*) that insulin treatment of adipocytes did not alter total mitochondrial calcium but caused an increase in the labile form of the cation and a decrease in the stable form. This change in calcium pools could be responsible for activating the phosphatase. This model is also consistent with the demonstrated ability of the material in fraction 2 to stimulate phosphoprotein phosphatase activity (*6*).

Our studies support the suggestion that the factor isolated from insulin-treated muscle (*6*) is the chemical mediator for insulin action. Another possible candidate for this role, H_2O_2 (*8*), has been tested in the adipocyte subcellular system and found not to stimulate pyruvate dehydrogenase activity (data not shown). An interesting correspondence between the activity of the material in fraction 2 (*6*) and of insulin, concanavalin A, and antibody to insulin receptor in the adipocyte subcellular system (*4*) is their diminished effect at high concentrations. This behavior has also been reported for an insulin-dependent cytoplasmic material from liver which stimulates Ca^{2+} uptake by isolated mitochondria (*9*). Further studies are necessary to isolate and characterize the chemical mediator generated by insulin in the adipocyte subcellular system in order to determine whether it is identical to the insulin-generated material isolated from muscle (*6*) or that from liver (*9*), or both.

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Generation by Insulin of a Chemical Mediator That Controls Protein Phosphorylation and Dephosphorylation

Abstract. *Deproteinized skeletal muscle extracts free of major nucleotides from control and insulin-treated rats were fractionated and assayed for inhibition of protein phosphorylation by cyclic adenosine monophosphate (AMP)-dependent and -independent protein kinases. A differential effect of insulin on a particular fraction was observed on cyclic AMP-dependent protein kinase but not on cyclic AMP-independent protein kinases. This fraction that inhibited cyclic AMP-dependent protein kinase also stimulated glycogen synthase phosphoprotein phosphatase. It is proposed that this fraction may contain a mediator substance generated in the presence of insulin.*

A chemical intermediate in the mechanism of action of insulin was proposed to explain the dissociation of insulin stimulation of glucose transport and glycogen synthesis (*1*). In keeping with this hypothesis, we have established two separate biochemical mechanisms for the activation of glycogen synthase in the absence and in the presence of a transportable hexose (*2*). Prior to this work we had already established that insulin activated glycogen synthase with

Table 1. Inhibition of cyclic AMP-dependent protein kinase by column fraction 2 from Sephadex G-25 chromatography. Column fraction 2 from control and insulin-treated rat skeletal muscle extracts was lyophilized and redissolved in 1 ml of 0.05N formic acid. The reaction mixture for the protein kinase assay contained (total volume, 90 μl) 5 μl of inhibitor fraction, 8 mM $MgCl_2$, 120 mM morphoethanesulfonic acid (MES) buffer (pH 6.6), 0.12 mM [γ-^{32}P]ATP (800 to 1000 cpm/pmole), 0.40 mg of histone per milliliter and, when present, 2.5 μM cyclic AMP. After 10 minutes at 30°C, 15-μl portions were pipetted onto instant thin-layer chromatography strips (ITLC; Gelman) that were then spotted with 20 percent trichloroacetic acid, 1 mM ATP, and 4 mM P_i. Strips were then chromatographed in 5 percent trichloroacetic acid containing 0.2M KCl, and analyzed for radioactivity (*12*).

Cyclic AMP	Inhibition of protein kinase (%)		Net effect due to insulin (%)
	Control	Insulin	
None	45.6	60.4	33
Present	52.1	63.0	21

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