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Intracellular site of insulin action: Mitochondrial Krebs cycle

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ABSTRACT **Effect of insulin on the oxidation of carbons-1,4 and -2,3 of succinate and their incorporation into protein were studied in isolated rat hepatocytes and diaphragm muscle pieces. Oxidation of carbons-2,3 of succinate and their incorporation into hepatocyte protein were stimulated significantly by insulin. Insulin had only a trivial effect on $^{14}CO_2$ formation from the carboxyl carbons of succinate. These data suggest that insulin affects only those carbons of succinate that are metabolized in the intramitochondrial Krebs cycle.**

A theory of the intracellular action of insulin that accounts for all of its anabolic effects on administration to whole animals or isolated cells or tissues postulated that insulin acts to connect hexokinase to the mitochondria of susceptible cells, thereby furnishing a respiratory control stimulus (1, 2). The anabolic processes, adjacent to the stimulated mitochondria (Fig. 1), receive an increase in energy (ATP) supply and are accelerated. Evidence has been accumulated (3, 4) that supports this proposition, but two points have not been clarified until the present work. The first is that no clear and reproducible effect of insulin on mitochondrial oxidation has been reported, and the second is that the total oxygen consumption of the cell is accelerated only trivially by insulin (5), compared to the 20–30% acceleration by insulin of the rate of most anabolic reactions (6–8). This report concerns experiments that demonstrate an immediate effect of insulin on Krebs cycle oxidation, which is almost maximal within 30 sec.

In this communication we report that insulin administration to isolated liver cells and diaphragm muscle pieces regularly stimulates the oxidation of those carbons of succinate that can only be oxidized to CO_2 in a second or subsequent turn through the mitochondrial Krebs cycle. Insulin has little or no effect on CO_2 formation from those carbons that can be oxidized by extramitochondrial Krebs cycle reactions. These results provide clear evidence for a major effect of insulin on the mitochondrial Krebs cycle and an explanation for two deficient points in the hexokinase binding theory of insulin action.

MATERIALS AND METHODS

Animals. Fed male Sprague–Dawley rats (180–250 g) were anesthetized by intraperitoneal injection of pentobarbital (45 mg/kg of body weight). The femoral vein was exposed through a small incision in the skin, and sodium heparin (500 units/kg of body weight) was injected intravenously.

Liver Perfusion and the Preparation of Isolated Hepatocytes. Liver perfusion and the preparation of isolated hepatocytes was carried out by a method modified from Seglen (9) and described in detail earlier (10). The viability of cells so prepared was routinely 90–95%.

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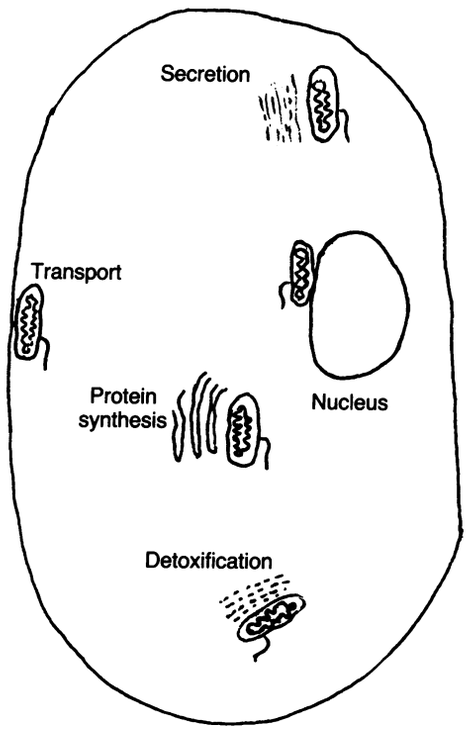

FIG. 1. Mitochondria as "outboard motors." Anabolic action of insulin on different sites in the cell.

Incubation of Hepatocytes. The medium consisted of 1.6 ml of Krebs–Henseleit bicarbonate (KHB) buffer (pH 7.4) containing all 20 natural amino acids, each to a final concentration of 0.5 mM. Incubation was begun with the addition of 0.4 ml of the cell suspension (approximately 8 mg of protein). After addition of cells, the flasks were flushed with O_2/CO_2, 95:5 (vol/vol), for 1 min and were stoppered. Incubations were carried out at 30°C in a Dubnoff metabolic shaker (60 oscillations per min). To the experimental flasks insulin (crystalline, bovine, Calbiochem) was added to a final concentration of 10 milliunits/ml at time zero, 15, 30, 45, and 60 min. At 60 min, isotope ([1,4-^{14}C]- or [2,3-^{14}C]succinate; ICN) was added in tracer quantities (1 μCi; specific activity, 15 mCi/mmol; 1 Ci = 37 GBq), and the flasks were fitted for incubation and CO_2 collection as described (10).

To study the immediate effect of insulin on the oxidation of [2,3-^{14}C]succinate, hepatocytes were preincubated for 5 min as above in the presence of 0.5 μCi of the isotope. Insulin (10 milliunits/ml) was added at 5 min, and incubations were terminated after 0.5, 1.0, 1.5, 2.0, and 5.0 min following the insulin addition by injecting 0.6 ml of 3 M perchloric acid into the flasks. CO_2 was collected as described (10).

Incubation of Diaphragm Pieces. Rat diaphragm pieces were isolated as described (11) and were incubated at 30°C in

Abbreviation: *P-enol*pyruvate, phospho*enol*pyruvate.

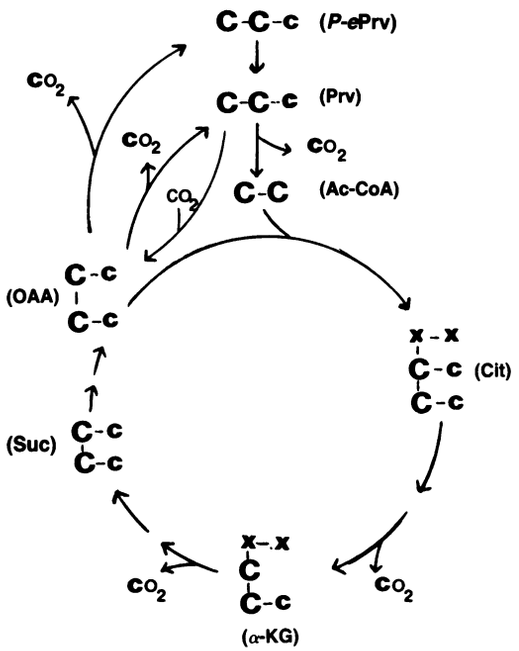

FIG. 2. Metabolic disposal of carbons-1,4 and -2,3 of succinate. C, aliphatic carbons; c, carboxyl carbons; x, acetate carbons reentering the Krebs cycle; Prv, pyruvate; *P-e*Prv, *P-enol*pyruvate; Cit, citrate; α-KG, α-ketoglutarate; OAA, oxaloacetate; Suc, succinate.

KHB buffer containing 5 mM glucose. Insulin and isotope were added at time zero, and incubations were terminated at 30 min by injecting 0.6 ml of 3 M perchloric acid into the flasks.

Protein Assay and the Determination of Radioactivity. After the perchloric acid acidification, the contents of the flasks were transferred to test tubes and centrifuged at 900 × *g* for 10 min. The resulting precipitates were resuspended in 0.7 M percholic acid containing 10 mM succinic acid and recentrifuged. The precipitates were processed for the determination of protein radioactivity and assay as described (10). Protein was estimated by the method of Lowry *et al.* (12).

Calculations. Data are expressed as specific activity (cpm/mg of protein). All values are presented as means ± SEM. Data were analyzed with the paired Student *t* test.

RESULTS

During the incubation period of 2 hr, the hepatocytes maintained their viability. Trypan blue exclusion tested at 120 min was about 90%.

Effect of Insulin on the Oxidation of Carbons-1,4 and -2,3 of Succinate. The pathways for the oxidation of carbons-1,4 and -2,3 of succinate are presented in Fig. 2. Table 1 shows that the activity of $^{14}CO_2$ produced from [1,4-^{14}C]succinate was about 10-fold higher than that from [2,3-^{14}C]succinate. Insulin increased the $^{14}CO_2$ production from [2,3-^{14}C]succinate significantly (29.8%, $P < 0.001$) and had a minimal effect on the $^{14}CO_2$ formation from carbons-1,4. Over a 1-hr period of incubation with either form of labeled succinate, $^{14}CO_2$ production was almost linear. Hepatocytes preincubated with isotope for 5 min showed an increase in $^{14}CO_2$ production within 30 sec after the addition of insulin (Fig. 3). The increase in rate caused within 30 sec was about the same as the increase in rate caused by insulin at longer time intervals. There was no significant effect of insulin on the oxidation of carbons-1,4 of succinate at any time point.

Effect of Insulin on the Oxidation of Succinate Carbons by Isolated Rat Diaphragm Pieces. Activity of $^{14}CO_2$ produced from [1,4-^{14}C]succinate was 4- to 5-fold higher than from [2,3-^{14}C]succinate (Table 2). Insulin increased the $^{14}CO_2$ formation from [2,3-^{14}C]succinate by about 34% ($P < 0.005$) and only about 4% from [1,4-^{14}C]succinate.

Effect of Insulin on the Incorporation of Succinate Carbons-1,4 and -2,3 into Hepatocyte Protein. Succinate carbons can be incorporated into protein by the transamination of oxaloacetate, pyruvate, or α-ketoglutarate through the amphibolic reactions of the Krebs cycle. Insulin caused a significant increase in the incorporation of succinate carbons from all positions into hepatocyte protein (Table 3). Although the specific activities of the tracers used were the same, the radioactivity incorporated into protein from [2,3-^{14}C]succinate was about twice as great from [1,4-^{14}C]succinate. The incorporation of tracer carbons into protein was linear over the period of 1 hr for both labels.

DISCUSSION

In order to be metabolized, the succinate molecule has to enter the mitochondrion because of the exclusive inner mitochondrial membrane locations of succinic dehydrogenase (13). In these experiments, more than 90% of the added tracer succinate was metabolized and recovered in various metabolic components (14, 15).

Data presented in Table 1 show that $^{14}CO_2$ produced from [1,4-^{14}C]succinate is about 10-fold higher than that produced from [2,3-^{14}C]succinate. Carbon-1 and carbon-4 of succinate have two general pathways for oxidation to CO_2. Succinate molecules leaving the Krebs cycle as fumarate, malate, or oxaloacetate can lose either carbon-1 or -4 in the phospho*enol*pyruvate (*P-enol*pyruvate) carboxykinase or oxaloacetate decarboxylase reactions (Fig. 2) to become labeled *P-enol*pyruvate or pyruvate. When [1-^{14}C]pyruvate is decarboxylated in the pyruvate dehydrogenase reaction, it loses its other carboxyl carbon derived from succinate, and only carbon-2 and -3 of the original succinate molecule reenter the Krebs cycle as acetyl-CoA. On the other hand, if the oxaloacetate derived from succinate remains within the mitochondrion and condenses with acetyl-CoA to become citrate, the carboxyl carbons will be lost as CO_2 in the isocitrate dehydrogenase and α-ketoglutarate dehydrogenase reactions. Carbon-2 and -3 of the original succinate molecule

Table 1. Effect of insulin on the oxidation of succinate carbons-1,4 and -2,3 by isolated hepatocytes

	$^{14}CO_2$, cpm × 10^{-2}/mg of hepatocyte protein		Percentage increase	Significance
	Control	Insulin		
[1,4-^{14}C]Succinate	1029.19 ± 41.33	1126.8 ± 52.0	9.9	NS
[2,3-^{14}C]Succinate	99.30 ± 5.75	128.9 ± 6.83	29.8	$P < 0.001$
Ratio 1,4/2,3	10.32	8.74		

Values are means ± SEM ($n = 11$). NS, not significant (by paired *t* test). Incubation conditions were as follows: 1.6 ml of KHB buffer (pH 7.4) containing 0.4 ml of cell suspension (approximately 8 mg of protein) and 0.5 mM each of 20 natural amino acids was incubated without insulin or with insulin (10 milliunits/ml) added at 0, 15, 30, 45, and 60 min. Isotope (1.0 μCi) was added at 60 min, and incubation was terminated at 120 min by the addition of 0.6 ml of 3 M PCA.

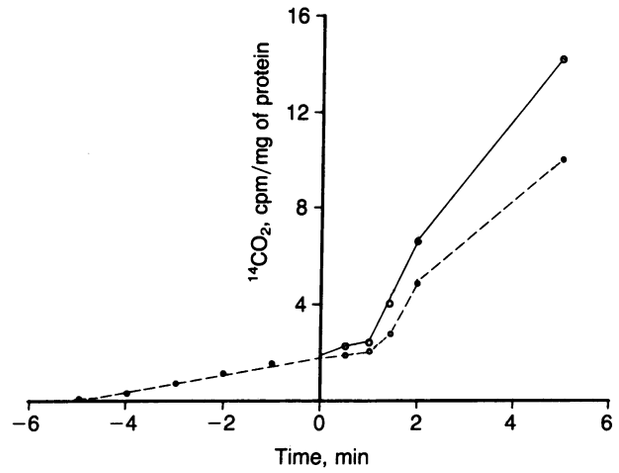

FIG. 3. Time course of insulin action on the oxidation of carbons-2,3 of succinate. Insulin was added at time zero. ——, Control; —, with addition of insulin.

are retained as carbon-1 and -2 of the succinate entering the second turn of the cycle. It takes at least two turns in the Krebs cycle to lose carbon-2 and three turns to lose carbon-3 of succinate as CO_2. Carbon-2 and -3 of succinate do not form CO_2 in the Krebs cycle reactions that occur in the cytosol.

Recent work from our laboratory (14, 16) has shown that about 70% of the labeled succinate carbons leave the Krebs cycle through the *P-enol*pyruvate carboxykinase or oxaloacetate decarboxylase reactions, 20–25% leave the cycle as glutamate (through transamination from α-ketoglutarate), and only about 5–10% of the total carbons entering as succinate remain within the mitochondrion to complete a second pass through the Krebs cycle. By applying the foregoing data to Fig. 2, it is clear that only these 5–10% of the carbons, which were labeled at positions 2 and 3, are sensitive to insulin action. Though only 5–10% of the total succinate carbons continue through the α-ketoglutarate dehydrogenase and succinyl thiokinase steps and complete a second pass in the Krebs cycle, insulin stimulates their traverse through the second pass by about 30%. The fact that 90% of the carbons are utilized suggests that a third pass of carbons-2,3 of succinate also takes place and is stimulated by insulin. Insulin has little or no effect on the oxidation of carbons-1,4 but does stimulate oxidation of carbons-2,3 of succinate by diaphragm muscle (Table 2). The ratio of $^{14}CO_2$ produced from [1,4-^{14}C]succinate to [2,3-^{14}C]succinate is lower perhaps because of the low activity of *P-enol*pyruvate carboxykinase in the muscle tissue. Most of the succinate carbons that were not oxidized to CO_2 were recovered in other metabolic components. Amino acids contributed to a very large percentage of the recovered radioactivity (14).

It has been suggested by several workers (17, 18) that insulin has only a delayed or a trivial effect on liver metabolism. Our data (Fig. 3) show that the effect of insulin on the oxidation of carbons-2,3 of succinate was an immediate one with the insulin-stimulated rate almost maximal within 30 sec.

Insulin stimulated the incorporation of carbons-1,4 and -2,3 into hepatocyte protein ($P < 0.001$). The radioactivity incorporated into protein from [2,3-^{14}C]succinate was almost twice as great as from [1,4-^{14}C]succinate. Analysis of the medium amino acids showed that more than 50% of the radioactivity recovered in the amino acid fraction was in glutamic acid, with alanine contributing about 17% and a trivial amount in aspartic acid (16). A greater incorporation of the labeled carbons-2,3 of succinate into hepatocyte protein and amino acids can be explained with the help of Fig. 2. Since 70% of the succinate carbons pass through the *P-enol*pyruvate carboxykinase or oxaloacetate decarboxylase reactions, at least one carbon (1 or 4) of succinate is lost in the conversion of oxaloacetate to pyruvate. Pyruvate is then transaminated to alanine, with only one of its carbons being radioactive. Neither carboxyl carbon ends up in glutamate by this route. On the other hand, if oxaloacetate condenses with acetyl-CoA to form citrate, only one of the radioactive carbons of succinate (1 or 4) will be incorporated into glutamate as carboxyl carbon. Only if oxaloacetate transaminates to aspartate can both carbon-1 and -4 be incorporated into protein. However, both carbon-2 and -3 of succinate can be incorporated into protein as alanine, serine, glutamate, or aspartate. CO_2 production from carbon-1 and -4 is much greater than from carbon-2 and -3, but protein incorporation of the carboxyl carbons is less than from the aliphatic carbons. We interpret this also to support the view that the effect of insulin must be on the mitochondrial cycle.

Insulin had a far greater stimulatory effect on the incorporation of carbon-2 and -3 of succinate into protein than carbon-1 and -4. We have shown that insulin stimulates the incorporation of [2-^{14}C]- and [3-^{14}C]pyruvate and [U-^{14}C]alanine into protein and has little effect on the incorporation of [1-^{14}C]pyruvate or [1-^{14}C]alanine, which lose their carboxyl carbons in the pyruvate dehydrogenase reaction (19, 20). The labeled carboxyl carbon of pyruvate can be incorporated into protein only via transamination to [1-^{14}C)alanine, the entry of which into protein is not stimulated by insulin. It has been shown that insulin stimulated the movement of pyruvate carbon-2 and -3 into protein primarily as glutamate (20).

The major finding of this study is the highly selective effect of insulin on the mitochondrial Krebs cycle. The differential conversion of carbons-1,4 and -2,3 show that more than 90% of the molecules that enter the cycle as succinate from the medium do not complete one full turn in the cycle and are recovered as several metabolites (amphibolites). CO_2 production from the carboxyl carbons of succinate can occur extramitochondrially by the following two sequences of reactions: (*i*) succinate → fumarate → malate → oxaloacetate → *P-enol*pyruvate + CO_2, then *P-enol*pyruvate → pyruvate → acetyl-CoA + CO_2; (*ii*) oxaloacetate → pyruvate + CO_2 and pyruvate → acetyl-CoA + CO_2. This oxidation can occur intramitochondrially through the citrate synthase reaction, losing CO_2 in the isocitrate dehydrogenase and α-ketoglutarate dehydrogenase reactions. This only requires a single turn of the Krebs cycle. CO_2 production from the aliphatic carbons of succinate can occur only in the second and third traverse of the Krebs cycle. Analysis of about 40 experiments

Table 2. Effect of insulin on $^{14}CO_2$ production from [1,4-^{14}C]succinate and [2,3-^{14}C]succinate by isolated diaphragm pieces

		$^{14}CO_2$, cpm × 10^{-2}/g of tissue		Percentage	
	n	Control	Insulin	increase	Significance
[1,4-^{14}C]Succinate	4	1246.2 ± 62.2	1301.03 ± 187.6	4.4	NS
[2,3-^{14}C]Succinate	5	239.4 ± 25.3	320.30 ± 34.5	34.1	$P < 0.005$
Ratio 1,4/2,3		5.2	4.06		

Values are means ± SEM; NS, not significant (by paired *t* test).

Table 3. Effect of insulin on the incorporation of succinate carbons-1,4 and -2,3 into isolated hepatocyte protein

	^{14}C incorporation, cpm $\times 10^{-2}$/mg of protein		Percentage increase	Significance
	Control	Insulin		
[1,4-^{14}C]Succinate	79.2 ± 6.25	90.14 ± 7.29	13.9	$P < 0.001$
[2,3-^{14}C]Succinate	112.5 ± 10.36	140.00 ± 10.9	24.5	$P < 0.001$

Values are means ± SEM (n = 11). Incubation conditions were as in Table 1.

under many conditions showed the mean percentage stimulation of CO_2 formation from the carboxyl carbons of succinate to be 9% and from the aliphatic carbons, 30%. Since insulin stimulates CO_2 production from the aliphatic carbons of succinate about 3 times as much as from the carboxyl groups and since much more net CO_2 formation occurs from the carboxyl groups, it is apparent that insulin has no effect on the extra mitochondrial, incomplete Krebs cycle. On the basis of the fact that about 10 times as much carboxyl carbons are oxidized to CO_2 as aliphatic carbons, the data suggest that, after succinate is oxidized by the mitochondrial succinic dehydrogenase, at least 70% of it enters the cytoplasm, where it passes through oxaloacetate liberating CO_2 to form pyruvate and then back to the Krebs cycle via the pyruvate dehydrogenase reaction. This results in the loss of the second carboxyl carbon as CO_2 but preserves both aliphatic carbons. These carbons end up primarily in glutamate. Ten percent of the succinate metabolized by the mitochondrial Krebs cycle recirculates through the cycle, which is so compartmented that there is little communication between it and the extramitochondrial enzymes. Insulin stimulates oxidation only through the mitochondrial cycle by about 30%. The small, almost statistically insignificant, stimulation of oxidation of the carboxyl carbons by insulin would amount to 30% of the 10% of succinate carbons that remain in the mitochondrial Krebs cycle, or a stimulation of 3% of the gross oxygen uptake. This is consistent with many observations. Experiments with specifically labeled alanine and pyruvate (20) have shown that insulin stimulates primarily the oxidation of those carbons that must pass more than one complete turn of the Krebs cycle. The above results with succinate are consistent with this evidence and establish the complete mitochondrial Krebs cycle as the site of stimulation by insulin.

The original theory of insulin action (2) postulated that all of the anabolic effects of insulin, including protein and carbohydrate synthesis as well as membrane transport, could be brought about by increasing "respiratory control" of the Krebs cycle in insulin-sensitive tissues by the insulin-mediated attachment of hexokinase to mitochondria. Although these anabolic effects have been verified (4), it has not been possible to show quantitatively equivalent effects on respiration and ATP generation. The present results coupled with further consideration of the disposal of energy by the tissues explain this discrepancy.

The anabolic activities of the cell, including transport of precursors of protein and glycogen, consume less than 10% of the gross ATP production of the resting cell. Most of the ATP produced is consumed in maintenance reactions such as Na^+/K^+-ATPase (21), which have never been shown to be influenced significantly by insulin (22). The insulin–hexokinase theory concerns this anabolic portion of the total ATP production. This theory described the mitochondria as small "outboard motors" generating and delivering energy at the sites of anabolic activity (3). The present results show that, indeed, insulin acts on a small compartment, the mitochondrial Krebs cycle, which appears to be directly coupled to the anabolic utilization of ATP.

In the original proposal, insulin was described as a linking peptide between hexokinase and specific sites in the mitochondrion (2). Certain compounds have been suggested as "second messengers" between a plasma membrane insulin receptor and the ultimate site of insulin action. Our earlier report (20) that insulin does not stimulate oxidation of the carboxyl carbon of pyruvate or alanine clearly shows that the compounds that have been suggested are not involved in the mechanism of insulin action, at least in liver, because the pyruvate dehydrogenase system, which is used to identify the proposed "second messengers," is not activated significantly even as insulin exerts strong effects on protein, fat, and carbohydrate synthesis. If the suggested "second messengers" were involved in insulin action, there should be proportional or greater increments in oxidation of carbon-1 of pyruvate or carbons-1,4 of succinate. A recent report by Marshall (23) shows that insulin indeed enters the cell; hence, it supports the possibility that it is indeed insulin that binds hexokinase to mitochondria (2).

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