The Effect of Insulin on Adipose Tissue

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THE RESULTS of a combined biochemical and electron microscopic study of the changes produced by insulin in the adipose cells of the epididymal fat pad of the rat *in vitro* are reviewed.[1-3] These experiments followed the observations of Winegrad and Renold[4] who demonstrated that the addition of insulin to the epididymal fat pad of the rat, incubated in bicarbonate buffered media, markedly stimulates the net reactions 1 to 3 (Fig. 1) whereby glucose is converted to fat. Reaction 4, a true respiratory one, is not stimulated by the addition of insulin.

$$\text{Reaction 1} = \text{Glucose} \rightarrow 2 \text{ acetyl CoA} + \text{DPNH} + 2\,CO_2 + 2\,P$$

$$\text{Reaction 2} = \text{Glucose} + 1\,P \rightarrow \text{pentose} - PO_4 + 2\,\text{TPNH} + CO_2$$

$$\text{Reaction 3} = n(\text{acetyl COA} + 2n\text{-}2\,(\text{TPNH} + \text{DPNH}) \rightarrow \text{Fatty acid}$$

$$\text{Reaction 4} = \text{Glucose} \rightarrow 6\,CO_2 + H_2O$$

FIG. 1. Unbalanced equation approximating the effect of insulin on the synthesis of lipid with a marked output of CO_2.

During the synthesis of fat from glucose, a marked excess of carbon dioxide output over oxygen consumption can be expected. The effect of insulin on the total exchange of gas in the epididymal fat pad was studied by Ball, Martin and Cooper[5] by means of the Warburg manometric apparatus. Paired pieces of epididymal fat pad, approximately 200 mg. in weight, were placed in two containers of bicarbonate buffered Ringer solution. One solution contained glucose, 4 mg./ml., and the other did not. During the first sixty minutes of the experiment a slight negative pressure change, indicating that oxygen consumption exceeded carbon dioxide production, was seen. The addition of insulin after sixty minutes to both solutions yielded a final concentration of 10^5 μU./ml. In the solution which contained glucose, a positive gas pressure response was evident within ten minutes and marked by twenty minutes after the addition of insulin. This meant the gas, presumably carbon dioxide, was evolving more rapidly than the oxygen was being consumed, and the respiratory quotient changed from a slight negative to a marked positive value. This evolution of gas was remarkably linear with time and was consistent with the expected increase in production of carbon dioxide (Fig. 1).

In a similar type of experiment, the amount of insulin was lowered and it was found that 1/100 of the initial quantity or 10^3 μU./ml. resulted in a consistent response almost equal (95 per cent) to that shown previously. No exchange of gas occurred in the absence of glucose and as little as 0.125 mg./ml. of glucose resulted in a positive response (slow and not linear with time). In other experiments, in which fat pads were placed in media containing glucose and insulin (added to one of the media), an identical result was obtained, indicating that in the absence of insulin the tissue appears unresponsive to glucose.

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Since this system offered the opportunity to compare possible ultrastructural changes in adipose cells with known metabolic changes in function, sections of incubated adipose tissue were examined under the electron microscope.

In order to be sure we were getting any biochemical effects of insulin, we used the identical procedure to monitor each experiment in which the tissue was examined with the electron microscope. The experimental tissues were removed from the containers ten, twenty, thirty, fifty and sixty minutes after the glucose and insulin were mixed, usually after twenty minutes, since by that time a marked response was evident. Control tissues, incubated in either glucose or insulin but not both, were taken at the end of the equilibration period, and at the same times as the experimental tissues. The small pieces of epididymal fat pad were fixed in cold 1 or 2 per cent osmium tetroxide, dehydrated in increasing concentrations of ethyl alcohol and embedded in butyl methacrylate. Thin sections, 3-400 Å in thickness, were cut with a Porter-Blum microtome and examined under an RCA EMU 2E electron microscope.

Mature yellow fat cells contained a huge droplet of lipid surrounded by a thin rim of cytoplasm. The cytoplasm contained occasional organelles, especially in the region of the nucleus which occurred in the thickest portion of the cytoplasm and bulged into the fat droplet. In the studies with the electron microscope, regions of cytoplasm were selected. Those regions where the cytoplasm occurred as an extremely thin structureless rim (1-2000 Å in diameter) were avoided, and those areas where the thickness of the cytoplasm was at least several times greater than 1-2000 Å and in which cytoplasmic organelles were numerous were studied.

In the specimens not subjected to insulin, the cytoplasm was homogeneous, dense, granular and contained some typical mitochondria. However, it contained only scanty reticulum, and practically no small droplets of lipid. In the experimental tissues subjected to insulin and glucose for a short period of time, a pronounced morphologic change was observed.

The plasma membrane was invaginated at many sites to form minute indentations. Numerous tiny vesicles were arranged in relation to the plasma membrane, suggesting that they might have been formed from a pinching off of a recessed tip of such a fold. Occasionally, deep membranous lined channels, connecting to the surface membrane, were apparent. Deeper in the cytoplasm, especially in the specimens that had been incubated for a longer period of time, numerous large, smooth, membrane-bound vesicles were seen. These vesicles appeared to form a discontinuous system of smooth reticulum from the plasma membrane and its infoldings to the interior of the cytoplasm. In addition, there was a loss of granularity of the cytoplasm and small droplets of lipid were found frequently. Although minute vesicles were seen bordering the plasma membrane in the control specimens (incubated with glucose but not insulin), they were sparse, and no cytoplasmic membranous system of vesicles or channels was found. Tissues, incubated with insulin in the absence of glucose, showed similar morphologic changes to tissues incubated in both solutions.

These morphologic changes, apparently caused by minute quantities of insulin, are reminiscent of the process of pinocytosis, described by W. H. Lewis[6] in 1931, in which the surface of the cell and adjacent cytoplasm are in a state of vigorous activity with an orderly flow of vesicles from the cell membrane to the interior of the cell. This type of membrane activation (flow) may be an important part of a type of active transport mechanism, which carries molecules from the surrounding medium (glucose) into the adipose cells. As such, this hypothesis fits the striking morphologic change which occurs with the addition of insulin to adipose tissue, involving the cell membrane, the formation of surface pinocytotic vesicles and a cytoplasmic membranous reticulum. Glucose is taken into the adipose cells and simultaneously converted into fat.

Whether or not the observed morphologic evidence of pinocytosis is sufficient to account for the rapid rate at which glucose enters the cell, is open to question. It is possible that, as a result of pinocytosis, structural changes may occur in the properties of the plasma membrane of the adipose cell, which result in a more rapid penetration of glucose into the cell. It should be

remembered that the process of vesicle formation results in a loss of surface membrane, requiring the cell to replace the membrane or to make some other adjustment. These studies lend support to the thesis, advocated by Levine et al.[7] and Park[8] that insulin acts by enhancing the entrance of glucose into the cells, and suggest a mechanism by which this may be accomplished.

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DISCUSSION

Dr. Lillian Recant (*St. Louis, Missouri*): Dr. Barrnett, did you have an opportunity to find out whether or not incubated growth hormone or prolactin produced similar changes on your cell membrane?

And secondly, do you have any comment to make about the type of studies Dr. Lacey has done, in which he has studied the effect of glucose infusions on the changes in the beta cells by electron microscopy? The changes, that he describes, in the beta cells look similar to me, because there is an invagination of the membrane and then this formation of droplets. Does this have to do simply with glucose penetrating into a cell, or is this an effect of insulin?

If you incubate fat tissue in glucose alone, little, if any, effect on the CO_2 mechanism is obtained and it is only after the addition of insulin or growth hormone that an acceleration of this process is seen.

Is this really an effect of insulin or could this be simply the phenomenon of glucose penetrating into the cell?

Dr. Barrnett: In answer to your first question, the experiments with prolactin are now in progress, and we are not quite ready to report them.

In answer to your second question, since glucose in the absence of insulin does not cause this morphologic evidence of stimulation at the surface of the cell, we believe, in this particular instance, which is a fairly narrow one, that insulin does stimulate the process of pinocytosis. We also believe, on the basis of the evidence of Holter and his co-workers on amoeba, that a whole hoard of substances will stimulate pinocytosis, and we have found in other instances, examples of this.

I do not think that glucose alone—and I can only say this about adipose tissue—stimulates pinocytosis.

A great many other things have been demonstrated to be carried into the cell by means of pinocytosis. It is difficult for electron microscopists to bring evidence to bear on any arguments that are concerned with soluble and diffusible compounds or ions. However, a large number of small molecules which are electron-opaque or contain electron-opaque atoms have been demonstrated to enter cells exactly by this means.

You may think that since these are solid particles, I am talking about phagocytosis. Where does phagocytosis end and pinocytosis begin? I think we are talking about the same thing.

Dr. Albert Winegrad (*Philadelphia, Pennsylvania*): Dr. Barrnett, first of all, have you found out whether or not some non-specific protein, for example, one of the serum proteins, would reproduce the effect you have described?

And secondly, if you prolong your incubation periods, can you observe pinocytosis?

Adipose tissue taken from normal rats can utilize glucose quite well. If you assume that this is an effect of insulin specifically, might it not just be stimulation of a process which should occur in this same tissue at sometime during a longer incubation period, let's say three hours, because we do know that fair quantities of glucose are taken up, oxidized to CO_2, and converted to fat in this time period.

Dr. Barrnett: Some other non-specific proteins such as protamine or lysozyme are at present being investigated.

In answer to the second point, the longest that we have prolonged our incubation for electron microscope examination was one hour. By this time, the tissue was quite extracted, so we looked no further. The results reported here are based on a comparison of adipose tissue, subjected to both insulin and glucose and of the same tissue of the same animal, subjected to only glucose.

Dr. Doolan (*Kalamazoo, Michigan*): Dr. Barrnett, do you know what happens to the membranes of other insulin-responsive tissues, such as muscle, as well as insulin-non-responsive tissues, presumably such as nervous tissue or, maybe, liver?

Dr. Barrnett: No, I do not. We have done some preliminary experiments on diaphragmatic tissue and liver and obtained some different results.

Dr. H. E. Wertheimer (*Jerusalem, Israel*): Have you pictures of starvation and feeding of carbohydrate?

Dr. Barrnett: Starvation alone causes such morphologic changes in fat that it confuses the picture. We tried this but it is just adding too many variables to the simple system that we have now. So we discarded it after trying two preliminary experiments.

Dr. Jay Tepperman (*Syracuse, New York*): Dr. Oscar Hechter at the Worcester Foundation told us he had been examining some adrenal cortical cells which

had been treated with ACTH, and he felt that the changes he was seeing in the cells of the adrenal cortex under the influence of ACTH stimulation were practically identical with the changes you have been describing.

I wondered whether you have seen his preparations. If so, can you comment on them for us?

DR. BARRNETT: Yes. I look forward to the day when it will be indicated that some hormones act on the cell surface.

DR. RACHMEIL LEVINE (*Chicago, Illinois*): Dr. Barrnett have you considered the suggestion made by Dr. Lazarow when Dr. Ball showed some of these pictures at Woods Hole this summer–the suggestion is in line with some of the earlier observations of Dr. Wertheimer–that is can these vesicles conceivably be glycogen, since the amount of water that had to be taken in would be enormous, and is this really a pocket of water, or is this glycogen prior to the formation of fat?

DR. BARRNETT: I do not believe that it is glycogen which displays a characteristic form in electron microscopic studies of a variety of organs. This form is granular and free in the cytoplasm instead of recurring in vesicles.

In other experiments, using goldthioglucose instead of glucose as our tag, we have found small dense bodies, presumably due to density of gold with the vesicles.

DR. JAMES SALTER (*Ontario, Canada*): What happens if you just add insulin alone?

DR. BARRNETT: The same thing. Insulin without glucose causes the same degree of pinocytosis. The pictures are such that you cannot tell one from the other.

DR. HERBERT S. ANKER (*Chicago, Illinois*): I believe there are values available for the rate of diffusion of water through cell walls. Can you calculate if the water intake is compatible with this exchange?

Furthermore, can you calculate the quantity of protein required in order to make the walls of your vesicles and see if that is compatible with the quantity of protein synthesis going on in these cells?

DR. BARRNETT: First of all, we have no idea, if insulin is stimulating this curious activity of the cell membrane, whether each vesicle that is formed depends upon a molecule of insulin or whether insulin stimulates a unit area of cytoplasm, which reacts in this way.

Secondly, we have no idea of the rate of the number of vesicles that are formed per given area of tissue. Other changes involving mitochondria and ground cytoplasm also occur in addition to pinocytosis, so that the degree of protein synthesis may not be a true measure.

DR. ANKER: Is there any way of spreading out the cell membrane by destroying the volume of the cells, so that you have a two-dimensional picture of the surface only, and then counting the pinholes, so to speak?

DR. BARRNETT: This could not be done easily. The process of pinocytosis as it occurs here, is one in which there are sites of extreme activity, moderate activity and no activity. To say that everything is going on at a uniform rate would be a mistake.

DR. LEVINE: I know of some experiments of Dr. Bloom at the National Institutes of Health showing that under the influence of insulin, tritium-labeled water does not enter faster into an insulin-sensitive tissue. This is one way of estimating whether the water is sufficient for this kind of process. Whether this is anything definitive or not, I do not know.

DR. BARRNETT: In our experiments, control tissues took up more water than the experimental ones. Remember that this also may be an excretory system as well as an intake system. There is no reason why a vesicle cannot make a roundtrip.

DR. JOHN R. BROBECK (*Philadelphia, Pennsylvania*) How do you know that the insulin is not inhibiting the dissolution of these things rather than stimulating the formation?

DR. BARRNETT: Without insulin, as in the control preparation, vesicles do not occur. You add insulin and they occur.

DR. BROBECK: This could be an inhibition.

DR. BARRNETT: I am assuming that lack of pinocytotic vesicles is an inhibition. Therefore, the presence of them is a stimulation.

DR. TEPPERMAN: I would like to get back to the earlier comment that was made about the possibility of all hormones eventually being shown to act by virtue of their effects on cell surfaces. We find that there are tremendous difficulties inherent in taking this position.

We have in mind the experiments of Randle, which are now becoming quite well known, in which the action of insulin is made to be analogous to certain effects of inhibitors, in anaerobiosis, or on the permeability of cells to glucose, for example. In the presence of anoxia or hypoxia, which are inhibitors of metabolism, glucose enters the cell more readily than it does in their absence.

Dr. Randle has made the intriguing suggestion that there is a "keeper-out-ase," if you will, of glucose, which is powered by some type of metabolic machinery in the cell, and that insulin may exert its effect by inhibiting or uncoupling the power from the "keeper-out-ase," thus permitting the glucose to get into the cell.

If there is any merit at all in this suggestion then there are all sorts of possibilities for hormones working on permeability mechanisms by actually exerting their effects on the machinery inside the cell that is coupled with the permeability mechanism.

It seems possible to me that in this particular tissue there may be deep kinds of effects of insulin inside the cell which would then express themselves in some of the changes which you may have seen. I am especially intrigued by the fact that you find these changes when you do not have glucose coming into the cell. The insulin works in the absence of glucose.

DR. BARRNETT: I do not see how the work of Dr. Randle bears on the present problem with which we are faced. Pinocytosis will probably end up being

stimulated by a great many things. One of the questions that is of import is the specificity of pinocytosis.

I am not surprised that anoxia will increase the degree in which materials get into the cell. Anoxia, for example, will increase the degree in which materials will traverse capillary beds.

On the one hand, we know that glucose is not getting into the cell without insulin and, on the other hand, we know that glucose is getting in with insulin. I believe, that there is a morphologic basis of function: and when I see this difference between the presence and absence of membranous activity of the plasma membrane, I am forced to attribute this thing to the change in function that has been observed.

DR. LEVINE: Dr. Barrnett, couldn't you resolve this problem of the functional connection of this morphologic change with the actual effect of insulin by doing exactly the same thing in a tissue the carbohydrate metabolism of which is not stimulated by insulin?

If pinocytosis does not occur then the insulin function and pinocytosis have something in common.

If pinocytosis still occurs, then I would be hard pressed to put the two things together.

The other thing, which I brought up when Dr. Ball presented some of the data, was to explain the specificity by which optical rotation can be linked with such an issue as pinocytosis.

DR. WINEGRAD: If we accept the work of Dr. Barrnett on the basis of his own interpretation, then I think we must make the assumption that the cell is perfectly capable of taking up glucose and oxidizing it to CO_2 in the absence of demonstrable pinocytosis, because in the particular preparation which he used, when the cell is incubated with glucose, there is no difficulty in demonstrating glucose utilization.

I would like to know how he resolves this conflict.

DR. BARRNETT: I did not realize the conflict existed. Maybe Dr. Renold could help me out on this point.

DR. ALBERT E. RENOLD (*Boston, Massachusetts*): It is just a matter of rate. It is true, speaking strictly, glucose does not get in unless there is pinocytosis. Yet there is difficulty associating that with the fact that if there is CO_2 production, glucose is getting in. But there may be a basal rate at which it can get through as well as a much faster rate following specific stimulation.

DR. J. A. F. STEVENSON (*Ontario, Canada*): Since you prefer the term pinocytosis to phagocytosis, it would seem to me that this implies that this mechanism is more concerned with the solvent than the solute. It might then follow that you think this is an important factor in the movement of water from one direction across the membrane to another.

Do you think that this process is involved in the movement of water, and have you looked at cells under conditions of acute hydration and dehydration?

DR. BARRNETT: I have not. My hunch would be that as the fluid medium, bathing the cell, enters it, pinocytosis may occur.

DR. F. X. HAUSBERGER (*Philadelphia, Pennsylvania*): If you incubate adipose tissue in one of the usual buffers, even with the addition of albumin, then the adipose tissue takes up water to the extent of about ten times the amount which is normally present in the adipose tissue. How does it get in without insulin?

DR. BARRNETT: I will just repeat the point that I made: that, as in the amoeba, maybe a whole hoard of substances will stimulate pinocytosis, in adipose tissue and in other cells.

DR. F. X. HAUSBERGER: If you incubate adipose tissue with glucose in a relatively low concentration, let us say 100 mg. per cent, and incubate another sample in 400 mg. per cent, there is a significant rise in CO_2 production and lipogenesis, due to the increase in the glucose concentration.

The increase is not so dramatic as if insulin was administered. Insulin increases it, maybe, six, eight, ten or twelve times. But still the increase may be twofold. Would there be any increased pinocytosis in this case?

DR. BARRNETT: I would say yes to this, just as a guess.