Effect of Insulin on Pyruvate Metabolism in Epididymal Adipose Tissue of the Rat

CORRELATION OF INTRACELLULAR PYRUVATE CONTENTS AND PYRUVATE DEHYDROGENASE ACTIVITY

By BRUCE G. BERMAN and MITCHELL L. HALPERIN
Department of Medicine, Medical Sciences Building, University of Toronto, Toronto, Ont., Canada

A method is described to measure the intracellular content of pyruvate and lactate in epididymal adipose tissue of the rat. The intracellular pyruvate concentration was approx. 330 μM. Intracellular pyruvate contents and the rates of pyruvate output were increased when $NNN'N'$-tetramethyl-*p*-phenylenediamine was added, and decreased in the presence of alanine. Insulin addition caused an increase in intracellular pyruvate contents only at the earlier time-period studied (1.5 min as against 20 min). Pyruvate dehydrogenase activity was increased in adipose tissue incubated *in vitro* with insulin. This increase occurred subsequent to the rise in the intracellular pyruvate content induced by insulin addition. The possible physiological implications are discussed.

Insulin increases the conversion of glucose into glyceride fatty acids in adipose tissue of the rat (Krahl, 1951; Winegrad & Renold, 1958). This observation was initially attributed primarily to an acceleration of the glucose-transport step (Jeanrenaud & Renold, 1959; Crofford & Renold, 1965). More recent investigations have revealed an additional site of action of insulin in the lipogenic pathway, independent of glucose transport, which augments pyruvate conversion into fatty acids (Jungas, 1970; Halperin, 1970). This insulin effect appears to be related to an increased activity of pyruvate dehydrogenase (Jungas, 1970, 1971; Coore *et al.*, 1971; Weiss *et al.*, 1971).

To evaluate the relative importance of these two sites of insulin action, it would be advantageous to correlate intracellular pyruvate concentrations with rates of flow through pyruvate dehydrogenase (see Rolleston, 1972). Indirect approaches utilized until now (Denton & Halperin, 1968; Hanson & Ballard, 1970; Saggerson & Greenbaum, 1970) have not provided measures of absolute amounts of pyruvate and have not been able to indicate rapid changes in pyruvate concentrations in adipose tissue. In the present paper we report a direct method for the measurement of the intracellular pyruvate concentration in adipose tissue under various metabolic conditions, and present information concerning the temporal relationship between the stimulation of glucose transport and pyruvate dehydrogenase activity by insulin.

Materials and Methods

Rats

Epididymal fat-pads were obtained from male Wistar rats (140–180 g) fed on a Purina Lab-Chow diet. In all experiments animals were allowed free access to feed before the time of killing (09:00 h). In any one experiment the rats were of the same age and similar weight (± 15 g).

Chemicals

Enzymes, cofactors, glycolytic intermediates and triethanolamine hydrochloride were obtained from Boehringer Mannheim Corp., New York, N.Y., U.S.A. ^3H_2O was from New England Nuclear Corp., Boston, Mass., U.S.A.; [U-^{14}C]sucrose and sodium [1-^{14}C]pyruvate were from Amersham/Searle Corp., Don Mills, Ont., Canada. Insulin was from Sigma Chemical Co., St. Louis, Mo., U.S.A. $NNN'N'$-Tetramethyl-*p*-phenylenediamine dihydrochloride was from British Drug Houses Ltd., Poole, Dorset, U.K., and silicone [Dow Corning, 200 Fluid, approx., 70×10^{-6} m^2/s (70 cSt) sp.gr. 0.934] from the Dow Corning Silicones Inter-America Ltd., Downsview, Ont., Canada. Anti-insulin serum was a gift from Dr. J. Logothetopoulos (University of Toronto).

Media

Fat-pads were preincubated and incubated in a bicarbonate-buffered medium (Krebs & Henseleit, 1932), gassed with $O_2 + CO_2$ (95:5). The concentrations of the various additions are given in the text or tables.

Procedures and methods

Incubation of epididymal fat-pads. The epididymal fat-pads were excised, rinsed and preincubated at 37°C for 30 min in medium containing glucose (10 mM) as described previously (Halperin, 1970). After preincubation the pads were removed, blotted lightly, weighed, cut into small (approx. 25 mg) pieces

and transferred into flasks containing 3 ml of incubation medium.

Separation and extraction of fat-pads from medium.
All procedures were carried out in 17 mm × 100 mm polypropylene tubes. After incubation the tissue and media were rapidly layered with 4 ml of precooled silicone, followed by addition of 2 ml of an ice-cold solution containing 70% (v/v) HClO$_4$-acetone-water (5:65:30, by vol.). The mixture was centrifuged at 600g for 5 min, and the top (HClO$_4$) layer, containing all the adipose-tissue fragments, was removed. The upper surface of the middle (silicone) layer was washed with fresh acetone–HClO$_4$ solution and this wash was added to the original supernatant fraction. The HClO$_4$ fraction, after homogenization, was neutralized to pH 7.0 with KOH and triethanolamine hydrochloride, and most of the acetone was removed under reduced pressure. A portion was analysed for radioactivity, and the remainder was passed through a Millipore filter before analysis for pyruvate and ATP.

Extraction and analysis of medium. The medium was carefully removed from below the silicone layer with a Pasteur pipette and transferred to a test tube containing 0.2 ml of 70% HClO$_4$. It was neutralized to pH 7.0 with saturated KHCO$_3$ before assay. Pyruvate and lactate were assayed fluorimetrically (Denton & Halperin, 1968). Plastic gloves were used to avoid contamination of samples.

Measurement of pyruvate dehydrogenase activity (EC 1.2.4.1). Paired epididymal fat-pads were preincubated for 30 min. They were then weighed and incubated for 10 min in buffer containing 1 munit of anti-insulin serum/ml. Insulin (10 munits/ml) was then added to the flask containing the left fat-pad. After further incubation, the pads were immediately frozen in liquid N$_2$, homogenized and assayed in quadruplicate for pyruvate dehydrogenase activity as described by Jungas (1971).

Measurement of extracellular and intracellular water. Water spaces were measured as described by Denton *et al.* (1966) by using [U-^{14}C]sucrose as the extracellular marker and ^{3}H$_2$O to measure the total water space.

Calculation of results. The volume of extracellular fluid accompanying the adipose-tissue fragments in a typical experiment was calculated as follows:

the acetone–HClO$_4$ phase (3.39 − 0.69 = 2.70 nmol/tissue sample or 11.9 nmol/g wet wt.).

Results

To evaluate the methods employed, the following studies were undertaken. Recovery of added pyruvate to the HClO$_4$–acetone upper phase was 97±3.02% (mean±S.E.M. for ten observations). Left and right pads from the same animals were always compared to avoid variations seen between individual animals. Duplicate treatments of right and left paired pads showed no significant difference in intracellular pyruvate contents (100±4.9%, mean±S.E.M. for seven observations). The left and right pads were randomized between control and experimental flasks. When the intracellular pyruvate contents were measured without using the silicone-layering technique, essentially similar amounts were obtained (9 nmol/g wet wt.). However, in the absence of silicone, the extracellular component was at least threefold larger (results not shown). ATP contents were also similar when measured in fat-pads frozen in liquid N$_2$ and by this silicone method (103±11 and 102±9 nmol/g wet wt. respectively).

The lactate and pyruvate amounts were measured in both the incubation medium and the tissue. The [lactate]/[pyruvate] ratio was significantly higher in the tissue than in the incubation medium (13.3±1.01 versus 8.62±0.58, $P < 0.02$, Table 1).

The intracellular pyruvate content in epididymal adipose tissue from normal fed rats was 9.88±0.85 nmol/g wet wt. The intracellular water space of white adipose tissue of rats used in these studies (120–140 g) was 30.0±1.4 μl/g wet wt. (mean±S.E.M. for 12 observations). This value is similar to that reported by Denton *et al.* (1966), Perry & Hales (1969) and Crofford & Renold (1965), and shows an intracellular pyruvate concentration of about 330 μM.

Effects of insulin on the intracellular pyruvate contents

In the presence of insulin there was a 49% increase in the intracellular pyruvate concentration after 1.5 min of incubation (Table 2). However, after 20 min the intracellular pyruvate concentrations were no longer elevated in the presence of insulin (Table 2).

$$\frac{\text{Total [U-}^{14}\text{C]sucrose (c.p.m. in acetone–HClO}_4\text{ phase)}}{\text{[U-}^{14}\text{C]Sucrose (c.p.m. per } \mu\text{l of incubation medium)}} = \frac{33\,460}{647.8} = 51.6\,\mu l$$

The pyruvate concentration in the incubation medium in this experiment was 0.0134 nmol/μl. Therefore the extracellular pyruvate content was 0.69 nmol (the product of 51.6 μl × 0.0134 nmol/μl). This value was then subtracted from the total pyruvate measured in

Effects of tetramethyl-p-phenylenediamine and alanine on intracellular pyruvate contents

The rates of fatty acid synthesis and pyruvate output into the incubation medium are increased by tetramethyl-*p*-phenylenediamine when adipose tissue

is incubated with glucose (Halperin, 1970; Saggerson, 1972). Under similar incubation conditions tetramethyl-p-phenylenediamine caused an increase in the intracellular pyruvate contents (Table 2).

Alanine addition has been previously found to inhibit lipogenesis in adipocytes from normal fed rats incubated with glucose and insulin, and to decrease rates of pyruvate output (Halperin, 1972). In the present series of experiments, intracellular pyruvate contents were significantly lower when alanine was added to the incubation medium (Table 2).

Effect of insulin on pyruvate dehydrogenase activity

Pyruvate dehydrogenase activity is increased when insulin is added to adipose tissue incubated *in vitro* (Jungas, 1970; Coore *et al.*, 1971; Weiss *et al.*, 1971; Taylor *et al.*, 1973 and Table 3). This effect was evident

Table 1. *Intracellular and media lactate and pyruvate contents*

Epididymal fat-pads were preincubated for 30min, then weighed, cut, and transferred into 3.0ml of fresh medium that contained glucose (10mm) and [U-^{14}C]sucrose (0.1μCi/ml), and incubated for 1.5min. Results are reported as means±S.E.M. for 24 observations.

	(nmol/g wet wt.)	
	Output into incubation medium	Intracellular content
Pyruvate	62.1 ± 3.3	6.37±0.48
Lactate	507.0 ±23.8	81.1 ±6.81
[Lactate]/[pyruvate]	8.62± 0.6	13.3 ±1.01

Table 2. *Effects of insulin, tetramethyl-p-phenylenediamine and alanine on intracellular pyruvate concentration*

Details of incubation conditions were as given in Table 1 with the following exceptions: at the beginning of the incubation, insulin (10munits/ml), tetramethyl-p-phenylenediamine (100μM) and alanine (2.5mm) were added in Expts. 1 and 2, 3 and 4 respectively. Results are reported as means±S.E.M. * $P<0.01$; ** $P<0.025$.

Experiment no.	Additions to incubation medium	Time of incubation (min)	Number of observations	Mean of intracellular pyruvate concn. (nmol/g wet wt.)	Effect of second addition (% of control)
1	Glucose	1.5	35	9.88±0.85	
	Glucose+insulin	1.5	35	13.91±1.29	+49.0± 8.76*
2	Glucose	20	16	18.76±2.93	
	Glucose+insulin	20	16	19.26±3.01	+0.3± 7.6
3	Glucose	5	17	11.16±1.27	
	Glucose+tetramethyl-p-phenylenediamine	5	17	15.96±1.75	+49.0±16.5*
4	Glucose+insulin	20	15	20.52±2.32	
	Glucose+insulin+alanine	20	15	16.0 ±1.54	−15.7± 6.6**

Table 3. *Effect of insulin on pyruvate dehydrogenase activity in adipose tissue*

Epididymal fat-pads were incubated as described in Table 1 and frozen in liquid N_2. The tissue was then homogenized and assayed for pyruvate dehydrogenase activity. Results are expressed as means ±S.E.M. All calculations are based on paired observations. * $P<0.01$; ** $P<0.05$.

Time (min)	Number of observations	Pyruvate dehydrogenase activity (nmol/2min per g wet wt.)		Effect of insulin (% of control)
		Control	+Insulin	
0.5	7	224±19	212±38	−2±11
1.0	9	200±27	197±31	−3± 8.0
1.5	10	217±22	213±23	0± 8.2
2.0	8	146±21	181±27	+22± 9.6**
3.0	10	196±36	221±38	+20±10.1**
5.0	8	143±23	191±24	+42±10*
20.0	7	156±32	283±54	+82±19*

after 2 min of incubation, and was pronounced after 5 min of incubation (Table 3).

Discussion

Any technique employed to measure the intracellular pyruvate content requires both a rapid separation of the adipose tissue from the incubation medium and a rapid arrest of tissue metabolism. The present paper describes a suitable method. The rapid separation from the incubation medium is required because, in a typical 1 h incubation, the quantity of pyruvate in the incubation medium is 15-fold higher than that in the tissue (approx. 150 nmol in the medium compared with an intracellular amount of approx. 10 nmol). Changes in tissue amounts would be difficult to detect because of this large extracellular quantity of pyruvate. The rapid separation of tissue from medium was achieved by centrifugation in a special system, in which the light adipose tissue, accompanied by very little extracellular fluid, floated through a silicone barrier and entered an ice-cold $HClO_4$ phase (in acetone to adjust its density to be less than that of the silicone).

Glucose deprivation during the isolation procedure seems unlikely, because the extracellular glucose accompanying the adipose tissue could sustain it for at least 5 min at maximum rates of glucose utilization (about 17 nmol/min per 100 mg wet wt.; Denton & Halperin, 1968). About 50 nmol of dissolved oxygen would have accompanied the adipose-tissue fragments that enter the $HClO_4$ phase, enough to sustain the oxygen consumption of 2 nmol/s per 100 mg wet wt. (Flatt & Ball, 1964) for at least 25 s. To assess whether there was significant tissue hypoxia during tissue separation, the [lactate]/[pyruvate] ratios were compared in tissue and medium. The [lactate]/[pyruvate] ratio was somewhat higher in the tissue (13.3) as compared with the incubation medium (8.6) (Table 1). This difference could represent either a slight degree of tissue hypoxia during the isolation procedure or could reflect the initial lower and subsequent higher lactate/pyruvate output ratios seen with insulin (M. L. Halperin, unpublished results). The concentrations of pyruvate and lactate are higher in cells than in the medium (Table 1 and B. G. Berman & M. L. Halperin, unpublished results), suggesting that these compounds may not be freely permeable across the adipocyte plasma membrane, and that variations in their relative plasma and medium concentration ratios might result from different permeabilities across the plasma membrane. A large concentration gradient for pyruvate was also found by Ballard & Hanson (1969) and Hanson & Ballard (1970), who reported tissue and medium pyruvate contents very similar to the values we obtained.

The rate of pyruvate output has been used in previous studies as an indicator of changes in intracellular pyruvate concentrations. On this basis, intracellular pyruvate contents were presumed to be raised by tetramethyl-p-phenylenediamine, and lowered by the presence of alanine in the incubation medium (Halperin, 1970, 1972). These inferences have now been confirmed by direct measurements (Table 2). Similar analogies were drawn from the results obtained when insulin was present; the rate of pyruvate output was initially very rapid but decreased appreciably with time. This could reflect the increased intracellular pyruvate amounts present only at the earliest time-period studied (Table 2 and B. G. Berman & M. L. Halperin, unpublished results).

The relative predominance of the two sites of action of insulin in the lipogenic pathway can now be evaluated. This is done by correlating the concentration of intracellular pyruvate with the rate of fatty acid synthesis (Rolleston, 1972). At the 20 min interval, activation of pyruvate dehydrogenase can be demonstrated to have an important role as there was increased flow through this step at a time when its substrate, pyruvate, was not increased. Direct demonstration of an increased pyruvate dehydrogenase activity at this time supports this conclusion (Table 3). In contrast, the intracellular pyruvate content was significantly increased by insulin at the 1.5 min interval. This suggests that insulin acceleration of the glucose-transport step was the dominant site of action of insulin at this time and is supported by the fact that pyruvate dehydrogenase activity was not yet demonstrably increased (Table 3).

In summary, intracellular pyruvate contents in epididymal adipose tissue incubated in vitro have been directly determined. These amounts are initially increased by insulin, and also by tetramethyl-p-phenylenediamine, whereas they are decreased by alanine. Longer periods of incubation with insulin (20 min) result in intracellular pyruvate concentrations approximately equal to the control value. These effects on intracellular pyruvate concentrations are consistent with the actions of insulin, tetramethyl-p-phenylenediamine and alanine on rates of fatty acid synthesis and changes in the activity of pyruvate dehydrogenase.

References

Ballard, F. J. & Hanson, R. W. (1969) *Biochem. J.* **112**, 195–202

Coore, H. G., Denton, R. M., Martin, B. R. & Randle, P. J. (1971) *Biochem. J.* **125**, 115–117

Crofford, O. B. & Renold, A. E. (1965) *J. Biol. Chem.* **240**, 14–21

Denton, R. M. & Halperin, M. L. (1968) *Biochem. J.* **110**, 27–38

Denton, R. M., Yorke, R. E. & Randle, P. J. (1966) *Biochem. J.* **100**, 407–419

Flatt, J. P. & Ball, E. G. (1964) *J. Biol. Chem.* **239**, 675–685

Halperin, M. L. (1970) *Can. J. Biochem.* **48**, 1228–1233

Halperin, M. L. (1972) *Can. J. Biochem.* **50**, 1326–1333

Hanson, R. W. & Ballard, F. J. (1970) *Biochim. Biophys. Acta* **201**, 196–204

Jeanrenaud, B. & Renold, A. E. (1959) *J. Biol. Chem.* **234**, 3082–3087

Jungas, R. L. (1970) *Endocrinology* **86**, 1368–1375

Jungas, R. L. (1971) *Metabolism* **20**, 43–53

Krahl, M. E. (1951) *Ann. N. Y. Acad. Sci.* **54**, 649–670

Krebs, H. A. & Henseleit, K. (1932) *Hoppe-Seyler's Z. Physiol. Chem.* **210**, 33–66

Perry, M. C. & Hales, C. N. (1969) *Biochem. J.* **115**, 865–871

Rolleston, F. S. (1972) *Curr. Top. Cell. Regul.* **5**, 47–75

Saggerson, E. D. (1972) *Biochem. J.* **128**, 1069–1078

Saggerson, E. D. & Greenbaum, A. L. (1970) *Biochem. J.* **119**, 221–242

Taylor, S. I., Mukherjee, C. & Jungas, R. L. (1973) *J. Biol. Chem.* **248**, 73–81

Weiss, L., Loffler, G., Schirmann, A. & Weiland, O. (1971) *FEBS Lett.* **15**, 229–231

Winegrad, A. I. & Renold, A. E. (1958) *J. Biol. Chem.* **233**, 267–282