Stimulation of Glucose Transport in Rat Adipocytes by Insulin, Adenosine, Nicotinic Acid and Hydrogen Peroxide

ROLE OF ADENOSINE 3':5'-CYCLIC MONOPHOSPHATE

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Glucose transport into adipocytes of the rat was measured by monitoring the conversion of [1-¹⁴C]glucose into ¹⁴CO₂. Glucose transport was made rate-limiting by increasing the flux through the pentose phosphate pathway with phenazine methosulphate, an agent that rapidly reoxidizes NADPH. Under these conditions, the observed rate of glucose disappearance from the incubation medium was about 20% higher than the rate of conversion of the C-1 of glucose into ¹⁴CO₂. Apparent rates of glucose transport were significantly increased by insulin, H₂O₂, adenosine and nicotinic acid. Stimulation of the apparent rate of glucose transport by insulin was dependent on adipocyte concentration, the hormone being most effective at relatively high cell concentrations. Adenosine and nicotinic acid further enhanced the maximum stimulation of glucose transport by insulin. Potentiation of insulin action by adenosine was more pronounced at lower cell concentrations. At relatively high cell concentrations the stimulatory action of insulin was markedly decreased by adenosine deaminase. Stimulation of apparent rates of glucose transport by the compounds noted above were antagonized by agents that increased intracellular cyclic AMP concentrations (theophylline and isoprenaline) and by dibutyryl cyclic AMP. Intracellular concentrations of cyclic AMP were significantly lowered when adipocytes were incubated with insulin, H₂O₂, adenosine or nicotinic acid. These effects were observed under basal conditions or when intracellular cyclic AMP concentrations were elevated by theophylline or isoprenaline. On the basis of the above data, we suggest that insulin, H₂O₂, adenosine and nicotinic acid may all stimulate glucose transport in rat adipocytes by lowering the intracellular cyclic AMP concentration. These data therefore support the hypothesis that cyclic AMP inhibits glucose transport in rat adipocytes.

We have previously described a method for the measurement of glucose transport in adipocytes that is simple, rapid and reproducible (Taylor *et al.*, 1976). The principle of the method is that glucose transport is made rate-limiting for [1-¹⁴C]glucose conversion into ¹⁴CO₂ in the pentose phosphate pathway by the addition of phenazine methosulphate to rapidly reoxidize NADPH (Katz & Wals, 1970). Using this method we previously suggested that insulin may stimulate glucose transport by lowering the intracellular cyclic AMP concentration (Taylor *et al.*, 1976).

In the present report, we have examined rates of glucose transport and cyclic AMP concentrations in rat adipocytes in the presence of various agents that mimic insulin action. The results indicate that stimu-

lation of glucose transport in rat adipocytes by insulin, adenosine, nicotinic acid or H₂O₂ is accompanied by a decreased adipocyte cyclic AMP concentration. These data further support the hypothesis that the intracellular cyclic AMP concentration is one of the factors controlling glucose transport in adipose tissue.

Materials and Methods

Chemicals

Enzymes, metabolic intermediates and cofactors were obtained from Boehringer/Mannheim Corp. (New York, NY, U.S.A.). Collagenase (EC 3.4.24.3) was obtained from Worthington Biochemical Corp. (Freehold, NJ, U.S.A.). Dowex 2 (8% cross-linked, 50–100 dry mesh), theophylline, phenazine methosulphate, isoprenaline (isoproterenol) hydrochloride,

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dibutyl cyclic AMP, nicotinic acid, adenosine, adenosine deaminase (EC 3.5.4.4), bovine serum albumin (fraction V) and catalase were supplied by Sigma Chemical Co. (St. Louis, MO, U.S.A.). H_2O_2 was from Ajax Chemicals (Sydney, Australia). D-[1- ^{14}C]-, D-[U- ^{14}C]- and D-[6- ^{14}C]-glucose and Hyamine hydroxide were obtained from Amersham/Searle Corp. (Don Mills, Ont., Canada). Crystalline bovine insulin was obtained from Commonwealth Serum Laboratories (Parkville, Australia). NaF was from BDH (Toronto, Ont., Canada).

Tissue preparation and incubation

Male Wistar rats (100–150g) of the John Curtin School strain or Canadian Breeding Labs (LaPrairie, Que., Canada) were allowed free access to laboratory chow until the time of killing (approx. 10:00h). In each experiment animals were of similar age and weight. Adipose tissue was prepared and incubated in a bicarbonate-buffered medium gassed with O_2/CO_2 (19:1) as described previously (Denton & Halperin, 1968). Adipocytes were prepared as described by Rodbell (1964) modified such that the glucose concentration was 1.67 mM. The cells were washed twice in glucose-free medium. Adipocyte cell diameter and number were estimated as described previously (Reardon *et al.*, 1973).

Extraction and analysis of adipocytes and incubation medium

Isolated cells or medium were extracted in 0.5M $HClO_4$ /ether for the measurement of metabolic intermediates or lipids as described previously (Denton & Halperin, 1968), except for cyclic AMP. For the measurement of intracellular cyclic AMP, the adipocytes were isolated by brief centrifugation (top speed, 30s, in a clinical centrifuge) and the floating adipocytes were treated with 2ml of 5% (w/v) trichloroacetic acid, mixed on a vortex mixer and left for 30 min at 0°C. After centrifugation (400g, 10 min), the supernatant was extracted with diethyl ether to remove the lipids and trichloroacetic acid, and then freeze-dried. The assay of cyclic AMP was performed with the kit, TRK 431, supplied by The Radiochemical Centre, Amersham, Bucks., U.K. (Brown *et al.*, 1971). Corrections for extracellular cyclic AMP were made by assaying cyclic AMP in the incubation medium and determining the amount of incubation medium in the adipocyte 'float' with radioactive sucrose as the extracellular marker. Total extracellular cyclic AMP was about 3% of the total under these experimental conditions.

In order to measure the quantity of radioactive glucose remaining in metabolic intermediates, a portion of the $HClO_4$ extract was neutralized with $KHCO_3$ and the precipitate removed by centrifugation at 2000g for 5 min in a bench centrifuge. Portions were taken both from zero-time controls and

after 45 min of incubation. The clear supernatant was transferred to a column (12cm $\times$ 1.5cm) of Dowex 2 (Cl^- form) and extensively washed with distilled water (1.5 litres). The intermediates were eluted with 100ml of 1M-HCl, and a portion was counted for ^{14}C radioactivity. Preliminary experiments demonstrated virtually complete separation of glucose and glucose 6-phosphate, and recovery of the latter intermediate on elution from the column with HCl.

Assay of [1- ^{14}C]glucose conversion into $^{14}CO_2$ in the presence of phenazine methosulphate

Adipocytes were incubated for 40 min in a bicarbonate-buffered medium containing 0.25 mM-glucose, 10 mM-NaF and 30 μ M-phenazine methosulphate as described previously (Taylor *et al.*, 1976). Final adipocyte concentrations are listed in Tables or text. Incubations were terminated by the addition of 0.8 ml of 0.5M- $HClO_4$. The $^{14}CO_2$ was trapped on filter paper impregnated with Hyamine hydroxide contained in suspended centre wells.

Radioactive assay

^{14}C radioactivity was assayed in a Packard model 3003 liquid-scintillation system, by using toluene-based scintillator containing 2,5-diphenyloxazole and 1,4-bis-(5-phenyloxazol-2-yl)benzene as previously described (Denton & Halperin, 1968). Quenching corrections were applied from the channels ratio method by using an external standard.

Results

Comparison of the rate of glucose uptake and $^{14}CO_2$ production

In experiments where [1- ^{14}C]glucose was the substrate, the rate of $^{14}CO_2$ production in the presence of phenazine methosulphate was linear over the experimental period and could account for most of the radioactivity in glucose that disappeared from the incubation medium. When [U- ^{14}C]glucose was used, the rate of $^{14}CO_2$ production was 3-fold higher than with [1- ^{14}C]glucose (Fig. 1). By contrast, less than 20% of the radioactivity in [6- ^{14}C]glucose that was taken up from the incubation medium appeared as $^{14}CO_2$. In the experiments with [U- ^{14}C]- and [6- ^{14}C]glucose, a significant fraction of the radioactive carbon that was taken up from the incubation medium could be found in triacylglycerols and phosphate esters (Table 1). Under these conditions, the production of pyruvate and lactate was virtually undetectable.

Effect of insulin, adenosine, nicotinic acid and H_2O_2 on apparent rates of glucose transport

When relatively low concentrations of adipocytes were used, the apparent rate of glucose transport was further enhanced in the presence of adenosine, both

in the presence and absence of insulin (Fig. 2). Adenosine was effective over a range of concentrations, with maximal stimulation occurring at 10 μ M. Under these conditions, the apparent rate of glucose transport was increased by only 67% by insulin (40 ng/ml) when added alone, but by over 200% when added together with 10 μ M-adenosine.

Similar potentiation of insulin action by adenosine is evident from the experiments detailed in Fig. 3. At low concentrations of adipocytes, insulin increased the rate of conversion of [1- 14 C]glucose into 14 CO $_2$ by only a small extent when examined over a range of hormone concentrations. The addition of 10 μ M-adenosine, however, greatly increased the response to insulin at hormone concentrations above 0.4 ng/ml. An increased response to insulin was also observed when higher adipocyte concentrations were used

(Figs. 4 and 5). Near-maximal effects of insulin (40 ng/ml) were attained at an adipocyte concentration of 2×10^4 cells/ml (Fig. 4). At cell concentrations above this value, 10 μ M-adenosine still enhanced the effect of insulin, but the effect was markedly decreased (Figs. 4 and 5). On the other

Fig. 1. Effect of insulin on 14 CO $_2$ production from [1- 14 C]- and [U- 14 C]-glucose

For details, see Table 1. Insulin, where present, was added to a final concentration of 20 ng/ml. Results are the means $\pm$ S.E.M. for five separate experiments, each assayed in triplicate. —, [U- 14 C]Glucose; ----, [1- 14 C]glucose; ●, no insulin; ○, insulin.

Fig. 2. Effect of adenosine on [1- 14 C]glucose conversion into 14 CO $_2$

Adipocytes (7600 ± 650 cells/ml) prepared from normal fed rats were incubated in a bicarbonate-buffered medium for 40 min as described in the Materials and Methods section. When present, insulin was added to a final concentration of 40 ng/ml. In the absence of further additions, the rate of [1- 14 C]glucose conversion into 14 CO $_2$ was 1.4 ± 0.3 μ g-atoms of glucose carbon/40 min per 10^7 cells. This value was normalized to 100 and all other values were expressed relative to this control. Results are reported as means $\pm$ S.E.M. for four separate experiments, each performed in triplicate. ○, Adenosine; ●, no additions.

Table 1. Fate of [14 C]glucose in adipocytes incubated with phenazine methosulphate

Isolated adipocytes (40–60 mg of lipid) were incubated in 2 ml of bicarbonate-buffered medium at 37°C for 45 min. The concentrations of additions were as follows: glucose, 0.25 mM; insulin, 20 ng/ml; phenazine methosulphate, 20 μ M; NaF, 10 mM. The incubation was stopped by the addition of 0.8 ml of 0.5 M-HClO $_4$ and the 14 CO $_2$ trapped on filter paper impregnated with Hyamine hydroxide. Results are reported as means $\pm$ S.E.M. The numbers of separate experiments, each assayed in triplicate, are indicated in parentheses.

Metabolic process	[1- 14 C]Glucose (14)	[6- 14 C]Glucose (4)	[U- 14 C]Glucose (10)
Glucose uptake (μ mol/g of lipid)	2.50 ± 0.23	2.57 ± 0.20	2.08 ± 0.25
14 CO $_2$ production (μ g-atoms/g of lipid)	2.01 ± 0.18	0.13 ± 0.02	5.71 ± 0.58
Conversion into triacylglycerol (μ g-atoms/g of lipid)	<0.01	0.57 ± 0.08	1.30 ± 0.19
Phosphate esters (μ g-atoms/g of lipid)	<0.1	0.63 ± 0.02	2.85 ± 0.15

Fig. 3. Effect of adenosine and insulin on [1-¹⁴C]glucose conversion into ¹⁴CO₂

Cells were incubated as described in the legend to Fig. 2. The adipocyte concentration was 8400 ± 700 cells/ml. In the absence of further additions, the rate of [1-¹⁴C]glucose conversion into ¹⁴CO₂ was 1.6 ± 0.5 μg-atoms of glucose carbon/40 min per 10^7 cells. This value was normalized to 100, and all other values were expressed relative to this control. Results are reported as means $\pm$ S.E.M. for five separate experiments, each performed in triplicate. ○, Insulin; ●, no additions.

hand, in experiments where relatively high adipocyte concentrations were used the stimulatory effect of insulin was severely decreased when adenosine was removed from the medium by the addition of adenosine deaminase (Fig. 5). The data presented in Fig. 6 indicate that nicotinic acid also enhances basal and insulin-stimulated rates of glucose transport when low concentrations of adipocytes were used. Indeed from a comparison of the data in Figs. 3, 4 and 6, it is clear that nicotinic acid and adenosine at the concentrations tested are almost equally effective agonists of glucose transport. The similar effects of adenosine and nicotinic acid are also evident in Fig. 7. In those experiments, H₂O₂ (10–1000 μM) stimulated the apparent rate of glucose transport, with maximal stimulation being achieved at 100 μM-H₂O₂. Nicotinic acid or adenosine further enhanced the stimulatory effect of H₂O₂ at all concentrations of H₂O₂ tested.

Fig. 4. Effects of adenosine and insulin on [1-¹⁴C]glucose conversion into ¹⁴CO₂

Cells were incubated as described in the legend to Fig. 2 at the concentrations indicated. Adenosine or insulin was added to a final concentration of 10 μM or 40 ng/ml respectively. Each point represents a single value determined in triplicate. ●, No additions; □, insulin; ■, insulin + adenosine.

Correlation of apparent rates of glucose transport and intracellular cyclic AMP concentrations

Agents that increased the apparent rate of glucose transport in rat adipocytes (insulin, H₂O₂, adenosine and nicotinic acid) all produced small but significant decreases in intracellular cyclic AMP (Table 2). In general, an inverse correlation between rates of glucose transport and intracellular cyclic AMP concentration was evident. Thus the lowest cyclic AMP concentrations were observed in the presence of insulin, together with either adenosine or nicotinic acid, when correspondingly the highest rates of glucose transport were also observed. In addition, agents that inhibited apparent rates of glucose transport (theophylline and isoprenaline) produced substantial elevation of intracellular cyclic AMP concentrations. Inhibition of glucose transport and elevation of cyclic AMP concentrations by these compounds were both antagonized to various extents by insulin, H₂O₂, adenosine or nicotinic acid. The greatest degree of antagonism was observed with the addition of insulin together with either adenosine or nicotinic acid to cells treated with theophylline or isoprenaline. Under these conditions apparent rates of glucose transport were returned to greater than control values and cyclic AMP concentrations were greatly diminished. Total intracellular cyclic AMP concentrations in these experiments, however, were never decreased below control values. It is also of interest that inhibition of glucose transport induced

Fig. 5. Effect of adenosine, adenosine deaminase and insulin on $[1-^{14}\text{C}]$ glucose conversion into $^{14}\text{CO}_2$

Cells were incubated as described in the legend to Fig. 2. Adipocyte concentration was 53800 ± 5400 cells/ml. Adenosine or adenosine deaminase was present at a final concentration of $10\ \mu\text{M}$ or $2\ \mu\text{g}$ of protein/ml respectively. In the absence of any additions, the rate of $[1-^{14}\text{C}]$ glucose conversion into $^{14}\text{CO}_2$ was $1.8 \pm 0.4\ \mu\text{g}$ -atoms of glucose carbon/40 min per 10^7 cells. This value was normalized to 100 and all other values were expressed relative to this control. Results are reported as means $\pm$ S.E.M. for five separate experiments, each performed in triplicate. $\circ$, Adenosine; $\blacksquare$, adenosine deaminase; $\bullet$, no additions.

Fig. 7. Effect of H_2O_2 on $[1-^{14}\text{C}]$ glucose conversion into $^{14}\text{CO}_2$

Cells were incubated as described in the legend to

Fig. 6. Effect of nicotinic acid and insulin on $[1-^{14}\text{C}]$ glucose conversion into $^{14}\text{CO}_2$

Cells were incubated as described in the legend to Fig. 2. Adipocyte concentration was 8600 ± 850 cells/ml. Nicotinic acid was added to a final concentration of $200\ \mu\text{M}$. In the absence of any additions, the rate of $[1-^{14}\text{C}]$ glucose conversion into $^{14}\text{CO}_2$ was $1.6 \pm 0.3\ \mu\text{g}$ -atoms of glucose carbon/40 min per 10^7 cells. This value was normalized to 100 and all other values were expressed relative to this control. Results are reported as means $\pm$ S.E.M. for four separate experiments, each performed in triplicate. $\blacksquare$, Nicotinic acid; $\bullet$, no additions.

by dibutyryl cyclic AMP was only marginally reversed by addition of insulin alone or in combination with adenosine or nicotinic acid.

Similar results were obtained when lipolytic rates were determined in these experiments (Table 2). Thus theophylline- or isoprenaline-stimulated rates of lipolysis were antagonized by insulin, H_2O_2 , adenosine or nicotinic acid. Insulin, in combination with either nicotinic acid or adenosine, was particularly effective, when lipolytic rates were lowered to control values. In contrast, lipolysis stimulated by

Fig. 2. Adipocyte concentration was 9900 ± 800 cells/ml. Adenosine and nicotinic acid were present at final concentrations of $10\ \mu\text{M}$ and $200\ \mu\text{M}$ respectively. In the absence of any additions, the rate of $[1-^{14}\text{C}]$ glucose conversion into $^{14}\text{CO}_2$ was $1.5 \pm 0.4\ \mu\text{g}$ -atoms of glucose carbon/40 min per 10^7 cells. This value was normalized to 100 and all other values were expressed relative to this control. Results are reported as means $\pm$ S.E.M. for four separate experiments, each performed in triplicate. $\circ$, Adenosine; $\blacksquare$, nicotinic acid; $\bullet$, no additions.

Table 2. *Effects of agents on apparent rates of glucose transport as measured by effects on rates of conversion of [1-¹⁴C]glucose into ¹⁴CO₂, intracellular concentrations of cyclic AMP and glycerol release*

Incubation details were as described in the legend to Fig. 1. Adipocyte concentration was $68\,000 \pm 8000$ cells/ml. In experiments where cyclic AMP concentrations were measured, the incubation time was decreased to 10 min. Where indicated, the following agents were present at the concentrations shown; insulin (40 ng/ml), H₂O₂ (100 μ M), adenosine (10 μ M), nicotinic acid (200 μ M), isoprenaline (10 μ M), theophylline (800 μ M) and dibutyryl cyclic AMP (1 mM). In the absence of further additions, the rate of conversion of [1-¹⁴C]glucose into ¹⁴CO₂ was 1.7 ± 0.4 μ g-atoms of glucose carbon/40 min per 10^7 cells, and the cyclic AMP concentration was 44 ± 6 pmol/ 10^7 cells. These control values were normalized to 100, and all other values were expressed relative to their respective control. Results are reported as means $\pm$ S.E.M. for six separate experiments where ¹⁴CO₂ and medium glycerol were measured and as means $\pm$ S.E.M. for four separate experiments where cyclic AMP was determined. * $P < 0.01$; ** $P < 0.05$ compared with no additions; † $P < 0.01$; †† $P < 0.05$ for effect of insulin.

Addition to incubation medium	Relative rate of [1- ¹⁴ C]glucose $\rightarrow$ ¹⁴ CO ₂		Relative intracellular cyclic AMP concn.		Glycerol release (μ mol/40 min per 10^7 cell)	
	–Insulin	+Insulin	–Insulin	+Insulin	–Insulin	+Insulin
None	100	168 $\pm$ 13*†	100	79 $\pm$ 6**	2.3 $\pm$ 0.2	2.0 $\pm$ 0.2
H ₂ O ₂	153 $\pm$ 12*	164 $\pm$ 11*	81 $\pm$ 5**	80 $\pm$ 6**	2.1 $\pm$ 0.3	1.9 $\pm$ 0.4
Adenosine	135 $\pm$ 8*	210 $\pm$ 22*†	89 $\pm$ 4**	72 $\pm$ 5**††	2.5 $\pm$ 0.5	2.0 $\pm$ 0.2
Nicotinic acid	128 $\pm$ 6*	194 $\pm$ 17*†	87 $\pm$ 3**	74 $\pm$ 5**	1.7 $\pm$ 0.2**	1.6 $\pm$ 0.3
Theophylline	53 $\pm$ 4*	71 $\pm$ 3*†	536 $\pm$ 38*	447 $\pm$ 21*	15.7 $\pm$ 0.6*	11.8 $\pm$ 0.4*†
Theophylline+H ₂ O ₂	86 $\pm$ 4*	89 $\pm$ 5*	373 $\pm$ 36*	396 $\pm$ 33*	13.9 $\pm$ 0.4*	12.3 $\pm$ 0.3*††
Theophylline+adenosine	103 $\pm$ 6	198 $\pm$ 16*†	186 $\pm$ 17*	114 $\pm$ 18††	6.5 $\pm$ 0.5*	2.3 $\pm$ 0.2†
Theophylline+nicotinic acid	96 $\pm$ 8	166 $\pm$ 11*†	174 $\pm$ 19**	108 $\pm$ 15††	3.2 $\pm$ 0.2**	2.4 $\pm$ 0.3
Isoprenaline	62 $\pm$ 4*	95 $\pm$ 5†	482 $\pm$ 26*	390 $\pm$ 30*	6.8 $\pm$ 0.4*	2.9 $\pm$ 0.2**†
Isoprenaline+H ₂ O ₂	100 $\pm$ 7	103 $\pm$ 7	406 $\pm$ 19*	384 $\pm$ 28*	4.0 $\pm$ 0.3*	2.7 $\pm$ 0.4††
Isoprenaline+adenosine	105 $\pm$ 4	168 $\pm$ 12*†	347 $\pm$ 22*	209 $\pm$ 25*††	2.8 $\pm$ 0.4	2.2 $\pm$ 0.5
Isoprenaline+nicotinic acid	108 $\pm$ 8	157 $\pm$ 10*†	369 $\pm$ 21*	216 $\pm$ 17*†	2.6 $\pm$ 0.3	2.1 $\pm$ 0.2
Dibutyryl cyclic AMP	59 $\pm$ 3*	74 $\pm$ 5*††	—	—	—	—
Dibutyryl cyclic AMP+adenosine	65 $\pm$ 6*	79 $\pm$ 4*††	—	—	—	—
Dibutyryl cyclic AMP+nicotinic acid	64 $\pm$ 4*	88 $\pm$ 3*†	—	—	—	—

dibutyryl cyclic AMP was only slightly decreased by any of these agents.

In experiments where NaF was omitted from the incubation medium, results qualitatively similar to those shown in Table 2 were obtained. However, in the absence of NaF, intracellular cyclic AMP concentrations were decreased by approx. 30% [39 ± 4 versus 25 ± 3 pmol/ 10^7 cells (means $\pm$ S.E.M.; $n = 6$; $P < 0.01$)], and the conversion of [1-¹⁴C]glucose into ¹⁴CO₂ was increased by 60% (1.5 ± 0.3 versus 2.4 ± 0.3 per 10^7 cells; $n = 8$; $P < 0.01$).

In the absence of fluoride, however, rates of conversion of [6-¹⁴C]glucose into ¹⁴CO₂ were increased from 0.04 ± 0.01 to 0.21 ± 0.05 μ g-atom of glucose carbon/40 min per 10^7 cells. This latter value is still only 9% of the corresponding value with [1-¹⁴C]glucose. Nevertheless, all data presented in this study were obtained in the presence of 10 mM-NaF.

Discussion

Comparison of the rate of glucose uptake and ¹⁴CO₂ production

In experiments with [1-¹⁴C]glucose as substrate, the rate of ¹⁴CO₂ production in the presence of

Scheme 1. Stoichiometry of the pentose phosphate pathway

phenazine methosulphate could account for most of the radioactivity in glucose that disappeared from the incubation medium (Table 1). On a theoretical basis, this was not expected because two molecules of unlabelled glucose 6-phosphate should be formed for every three molecules of [1-¹⁴C]glucose metabolized through the pentose phosphate pathway (Scheme 1) and should lead to a fall in the specific radioactivity of intracellular glucose 6-phosphate. The rate of ¹⁴CO₂ production is a function of both the specific radioactivity of glucose 6-phosphate and the flux rate through the pentose phosphate pathway. However, we had previously shown that glucose 6-phosphate concentrations were very low when phenazine methosulphate was added (Halperin *et al.*, 1978). It follows

that the rate of $^{14}\text{CO}_2$ production may not fall in this system if the rate of flux through the pentose phosphate pathway is considerably higher than the rate of glucose transport and the fall in glucose 6-phosphate specific radioactivity. This expectation seems to be correct, as demonstrated in the studies with $[\text{U-}^{14}\text{C}]\text{glucose}$ (Fig. 1, Table 1).

One would expect a much lower yield of $^{14}\text{CO}_2$ with $[\text{6-}^{14}\text{C}]\text{glucose}$, because the label could only reach the C-1 position of glucose 6-phosphate after several cycles of the pentose phosphate pathway (Scheme 1). Furthermore, since NaF was present in the incubation medium, enolase would be inhibited (Hewitt & Nicholas, 1963) and therefore little glucose carbon will be oxidized via pyruvate dehydrogenase or the citric acid cycle. In experiments with $[\text{U-}^{14}\text{C}]\text{glucose}$, the rate of $^{14}\text{CO}_2$ production in the presence of phenazine methosulphate was about 3-fold higher than that observed when $[\text{1-}^{14}\text{C}]\text{glucose}$ was the substrate in parallel flasks (Fig. 1, Table 1). When compared with the rate of glucose uptake, the rate of $^{14}\text{CO}_2$ production was also about 3-fold higher (Table 1), close to the predicted value for complete glucose oxidation in the pentose phosphate pathway as shown in Scheme 1. As in the experiments with $[\text{6-}^{14}\text{C}]\text{glucose}$, a significant fraction of radioactive carbon was found in triacylglycerol. In order to examine the fate of the remaining radioactive glucose carbon atoms, two additional products were examined. Little radioactive carbon was found in glycerol released into the incubation medium, whereas about 25% of radioactivity derived from $[\text{U-}^{14}\text{C}]\text{glucose}$ was found in organic phosphate esters in adipocytes and the incubation medium.

Some 80% of radioactive glucose was recovered in these studies. The most likely sources for the unmeasured radioactivity are glycogen, pyruvate, lactate or underestimates of organic phosphate esters. The reader should appreciate that this is a potential disadvantage for the use of the conversion of $[\text{1-}^{14}\text{C}]\text{glucose}$ into $^{14}\text{CO}_2$ with phenazine methosulphate to monitor the rate of glucose uptake. However, the simplicity of this technique and the low concentrations of glucose 6-phosphate in adipocytes are distinct advantages of this method.

Effects of insulin, adenosine, nicotinic acid and H_2O_2 on apparent rates of glucose transport

The previously reported 'insulin-like' actions of adenosine in adipose tissue include a decrease in hormone-stimulated adenylate cyclase activity, intracellular cyclic AMP concentrations and rates of lipolysis (Raben & Matsuzaki, 1966; Fain *et al.*, 1972; Fain, 1973; Ebert & Schwabe, 1973) and an increase in glucose utilization and oxidation (Dole, 1962; Schwabe *et al.*, 1974). The present study indicates that, in addition, adenosine stimulates glucose transport. Adenosine was most effective when added

together with insulin to low concentrations of adipocytes. At higher cell concentrations the effect of adenosine was diminished, whereas the response to insulin by itself was greatly increased. In addition, at high adipocyte concentrations, the effective removal of adenosine by treatment with adenosine deaminase severely decreased the response to insulin. In a previous study concerned with the effect of adenosine and insulin on lipolysis and glucose oxidation in rat adipocytes, responses to these agents showed a similar dependence on adipocyte concentrations (Schwabe *et al.*, 1974). In that study, it was proposed that the increased effectiveness of insulin at higher adipocyte concentrations resulted from an increased accumulation of adenosine. The results of the present study lend further support to this view and indicate that adenosine may play a role in the regulation of adipocyte metabolism.

Potential of insulin action in fat-cells was also observed in the presence of nicotinic acid (Fig. 6 and Table 2). Adenosine and nicotinic acid appeared almost equally effective in this regard (Figs. 3, 4 and 6 and Table 2), thereby raising the possibility that these two agents may share a common mechanism of action.

Apparent rates of glucose transport in rat adipocytes were also stimulated in the presence of H_2O_2 (Fig. 7 and Table 2). H_2O_2 has previously been reported to increase hexose transport in adipocytes by a mechanism involving oxidation of thiol groups (Czech *et al.*, 1974a,b). It has been suggested that similar oxidation of thiol groups may also be involved in the mechanism whereby insulin lowers cyclic AMP concentrations and stimulates glucose transport in rat adipocytes (Czech *et al.*, 1974b; Phelps & Varandani, 1977). Other similarities between the actions of insulin and H_2O_2 were also evident in Figs. 3, 6 and 7 and Table 2. Thus stimulation of apparent rates of glucose transport by either agent was further enhanced in the presence of adenosine or nicotinic acid, and the stimulatory effects of insulin and H_2O_2 were not additive and were both diminished in the presence of isoprenaline or theophylline. Moreover, when rates of glucose transport were increased in the presence of either insulin or H_2O_2 , intracellular cyclic AMP concentrations were decreased (Table 2). These data are consistent with the proposal that oxidation of thiol groups may be involved in the mechanism of action of insulin.

Correlation of apparent rates of glucose transport and intracellular cyclic AMP concentrations

In a previous paper (Taylor *et al.*, 1976) we reported an inverse correlation between apparent rates of glucose transport and intracellular cyclic AMP concentrations in rat adipocytes. In that study, apparent rates of glucose transport were inhibited by a range of agents (including lipolytic hormones and

theophylline) that elevated intracellular cyclic AMP concentrations. In contrast, insulin, nicotinic acid and clofibrate all lowered cyclic AMP concentrations and stimulated apparent rates of glucose transport. In the present study, two more compounds, reported to have 'insulin-like' actions in adipose tissue, H_2O_2 and adenosine, also increased apparent rates of glucose transport and lowered adipocyte cyclic AMP concentrations. In addition, adenosine and nicotinic acid further enhanced the effect of insulin on both these parameters (Figs. 2-6 and Table 2).

Potential of insulin action by nicotinic acid and adenosine was also evident when adipocytes were treated with theophylline or isoprenaline (Table 2). Under these conditions, although changes in the rate of glucose transport were still inversely correlated with changes in cyclic AMP concentration for any given absolute rate of glucose transport, total cyclic AMP concentrations were significantly higher than under control conditions. These data suggest, therefore, that if rates of glucose transport are strictly related to intracellular cyclic AMP concentrations in the presence of theophylline or isoprenaline, then a considerable proportion of the cyclic AMP produced in response to these agents must be located in a compartment without a major influence on the glucose-transport process. There is evidence suggesting that compartmentation of cyclic AMP does in fact occur within rat adipocytes. An earlier report suggested that a major portion of the cyclic AMP generated in rat adipocytes is in response to lipolytic hormones, and theophylline was not associated with the cyclic nucleotide pool assumed to influence lipolytic rates (Fain *et al.*, 1972). The data shown in Table 2 are also consistent with this proposal. Thus lipolytic rates stimulated by isoprenaline were decreased to control values by the addition of insulin together with either nicotinic acid or adenosine. However, as discussed above, cyclic AMP concentrations remained significantly higher than control values under these conditions. Furthermore, although rates of lipolysis in adipocytes are generally considered to be controlled by the intracellular cyclic AMP concentration (for review see Robison *et al.*, 1971), adipocyte cyclic AMP concentrations can be elevated by a number of agents (procaine, amitriptyline and phenethylbiguanide) without corresponding increases in lipolysis (D'Costa & Angel, 1975; Siddle & Hales, 1974; Lovrien *et al.*, 1972; Brown *et al.*, 1973). In contrast, glucose transport in rat adipocytes is inhibited by these agents (Taylor *et al.*, 1976). One possible explanation is that the cyclic AMP generated in response to these agents is located primarily in separate pools for the regulation of lipolysis and glucose transport.

General comments

The mechanism of action of cyclic AMP generally

involves phosphorylation of proteins with ATP as substrate (for review, see Robison *et al.*, 1971; Hollenberg & Cuatrecasas, 1975). Several reports indicate that membrane proteins are phosphorylated when adipocytes are incubated with lipolytic hormones (Avruch *et al.*, 1976; Forn & Greengard, 1976). Insulin reversed the effect of these agents on membrane phosphorylation (Forn & Greengard, 1976). In addition, glucose transport was inhibited in adipocytes after incubation with ATP (Chang & Cuatrecasas, 1974; Loten *et al.*, 1976; Halperin *et al.*, 1978). These findings led us to propose that the cyclic AMP-induced inhibition of glucose transport is mediated by a cyclic AMP-catalysed phosphorylation of membrane proteins (presumably the glucose transporter) by ATP (Taylor *et al.*, 1976; Halperin *et al.*, 1978).

The results reported are consistent with the above hypothesis. However, it is possible that the effects on glucose transport reported in the present study are not mediated solely by changes in cyclic AMP concentration. Adenosine has been reported to inhibit protein kinase activity in a number of mammalian tissues (Miyamoto *et al.*, 1969; Kuo & Greengard, 1969; Kariya & Field, 1976). This effect, however, has not always been observed in rat adipocytes (Fain & Wieser, 1975). The data reported in Table 1 suggest that adenosine probably stimulates apparent rates of glucose transport in rat adipocytes largely by lowering cyclic AMP concentrations rather than by directly affecting protein kinase activity. Thus inhibition of glucose transport by agents that elevate cyclic AMP concentrations (isoprenaline, theophylline) was largely overcome in the presence of adenosine, whereas adenosine only marginally affected inhibition of glucose transport induced by dibutyl cyclic AMP.

Glucose transport in rat adipocytes has also been reported to be inhibited by intracellular glucose or glucose metabolites (Halperin *et al.*, 1978). However, under the conditions used in this study (in the presence of $30\mu\text{M}$ -phenazine methosulphate) concentrations of glucose metabolites were extremely low (glucose 6-phosphate concentrations less than $1\text{ nmol}/10^7$ cells) and thus probably not able to influence the glucose transporter.

In conclusion, the data presented support the hypothesis that cyclic AMP may play a role in the control of glucose transport in rat adipocytes. In particular, the results suggest that insulin and other agents able to mimic the action of this hormone may exert their effects by lowering the cyclic AMP concentration in the relevant intracellular compartment.

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