Interrelationship and Control of Glucose Metabolism and Lipogenesis in Isolated Fat-Cells

CONTROL OF PENTOSE PHOSPHATE-CYCLE ACTIVITY BY CELLULAR REQUIREMENT FOR REDUCED NICOTINAMIDE ADENINE DINUCLEOTIDE PHOSPHATE

By H. KATHER, M. RIVERA and K. BRAND
Max-Planck-Institut für Ernährungsphysiologie, Dortmund, Germany

(Received 17 December 1971)

By using inhibitors and stimulators of different metabolic pathways the interdependence of the pentose phosphate cycle and lipogenesis in isolated fat-cells was studied. Rotenone, which is known to inhibit electron transport in the respiratory chain, blocked glucose breakdown at the site of pyruvate dehydrogenase. Consequently, because of the lack of acetyl-CoA, fatty acid synthesis was almost abolished. A concomitant decrease in pentose phosphate-cycle activity was observed. Phenazine methosulphate stimulated pentose phosphate-cycle activity about five- to ten-fold without a considerable effect on fatty acid synthesis. The influence of rotenone on both the pentose phosphate cycle and lipogenesis could be overcome by addition of phenazine methosulphate, indicating that rotenone has no direct effect on these pathways. The decreased rate of the pentose phosphate cycle in the presence of rotenone therefore has to be considered as a consequence of decreased fatty acid synthesis. The rate of glucose catabolism via the pentose phosphate cycle in adipocytes appears to be determined by the requirement of NADPH for lipogenesis. Treatment of cells with 6-aminonicotinamide caused an accumulation of 6-phosphogluconate, indicating an inhibition of 6-phosphogluconate dehydrogenase. The rate of glucose metabolism via the pentose phosphate cycle as well as the rate of fatty acid synthesis, however, was not affected by 6-aminonicotinamide treatment and could still be stimulated by addition of insulin. Since even in cells from starved animals, in which the pentose phosphate-cycle activity is extremely low, no accumulation of 6-phosphogluconate was observed, it is concluded that the control of this pathway is achieved by the rate of regeneration of NADP at the site of glucose 6-phosphate dehydrogenase.

In the preceding paper (Kather *et al.*, 1972) the dependence of the rate of fatty acid synthesis and of the pentose phosphate cycle on the amount of glucose uptake in fat cells was demonstrated. A correlation between lipogenesis and glucose metabolism via the pentose phosphate cycle was established. It was shown, however, that the amount of NADPH produced in this pathway was not sufficient to provide all the NADPH required for fatty acid synthesis.

There are numerous reports of a stimulation of the pentose phosphate cycle in various tissues by the addition of electron acceptors such as Methylene Blue or phenazine methosulphate, suggesting that the flux via this pathway might be controlled by mass action with respect to the NADP⁺/NADPH ratio (Brin & Yonemoto, 1958; Holzer & Witt, 1958; Roigas *et al.*, 1970; Brand *et al.*, 1970). No direct evidence for a control of the pentose phosphate cycle by an NADPH-requiring biosynthetic process has been presented so far. In the present paper experiments with inhibitors and stimulators of

different metabolic pathways are described, which provide evidence for a mass-action control of glucose metabolism in fat cells via the pentose phosphate cycle by cofactor limitation at the site of glucose 6-phosphate dehydrogenase.

Materials and Methods

Methods

Treatment of animals, incubation conditions, analytical procedures and calculations are described in the preceding paper (Kather *et al.*, 1972). For calculating the contribution of the pentose phosphate-cycle activity to glucose metabolism method A (Katz *et al.*, 1966) was employed.

Rotenone was added to the appropriate incubation media in an ethanol solution, to give a final concentration of 10 μ M. When present, the final concentration of phenazine methosulphate was 20 μ M.

In experiments with 6-aminonicotinamide, 3 g of adipose tissue was preincubated for 1 h in 4.5 ml of

Krebs–Henseleit bicarbonate buffer (Krebs & Henseleit, 1932), pH 7.4, in the presence of 0.65 mg of 6-aminonicotinamide. After addition of collagenase the incubation was continued for 1 h more and the cell preparation carried out as described in the preceding paper (Kather *et al.*, 1972). For enzymic determination of 6-phosphogluconate, perchloric acid extracts from incubation mixtures with glucose labelled in various positions with ^{14}C were combined and concentrated.

Chemicals

6-Aminonicotinamide was obtained from Fluka A.G., Buchs, Switzerland, rotenone from K & K Laboratories Inc., Plainview, N.Y., U.S.A., and phenazine methosulphate was a product of EGA Chemie, Steinheim, West Germany. All other chemicals used are listed in the preceding paper (Kather *et al.*, 1972).

Results

The effects of rotenone and phenazine methosulphate on glucose utilization, lactate and pyruvate production and on the incorporation of

^{14}C from glucose labelled in various positions into fatty acids, glyceride glycerol and CO_2 are shown in Table 1. Glucose uptake was only slightly increased by rotenone. The ratio of lactate to pyruvate released into the medium was raised from 4.4 in the absence to 11.1 in the presence of rotenone, reflecting a drastic shift of the cytoplasmic NAD^+/NADH couple to the reduced state. Of the glucose taken up 94% was converted into C_3 products (lactate + pyruvate + glyceride glycerol) when rotenone was present, whereas in the controls only about 50% was converted into C_3 compounds and a considerable amount was further degraded via the pyruvate dehydrogenase reaction to CO_2 and acetyl-CoA.

Consistent with these findings is the low yield of $^{14}\text{CO}_2$ from $[6\text{-}^{14}\text{C}]$ - and $[\text{U-}^{14}\text{C}]$ -glucose in the presence of rotenone. The diminished release of $^{14}\text{CO}_2$ from $[1\text{-}^{14}\text{C}]$ glucose further indicates that the pentose phosphate-cycle activity is also affected by rotenone. Of the $[\text{U-}^{14}\text{C}]$ glucose taken up 13% was incorporated into fatty acids in the controls, compared with 1.7% in the presence of rotenone. The incorporation of glucose label into glyceride glycerol,

Table 1. *Effect of rotenone and phenazine methosulphate on glucose uptake and utilization*

The amount of ^{14}C glucose converted into fatty acids, glyceride glycerol and CO_2 was calculated from the specific radioactivities used. The values from $[1\text{-}^{14}\text{C}]$ - and $[6\text{-}^{14}\text{C}]$ -glucose are expressed in terms of nmol of glucose, as if the amount of radioactivity measured was derived from $[\text{U-}^{14}\text{C}]$ glucose. The recovery was calculated from lactate and pyruvate production, determined enzymically, and from the conversion of $[\text{U-}^{14}\text{C}]$ glucose into fatty acids, glyceride glycerol and CO_2 . Experimental conditions and assays are described in the Materials and Methods section of the preceding paper (Kather *et al.*, 1972).

Conditions	Metabolism (nmol/2h per 100 μmol of triglyceride)			Glucose label	^{14}C Glucose incorpor- ated (nmol/2h per 100 μmol of triglyceride)			^{14}C Glucose recovery (%)
	Glucose utilization	Lactate production	Pyruvate production		Fatty acids	Glyceride glycerol	CO_2	
—	1140	560	127	1	160	189	245	81
				6	200	168	125	
				U	147	175	259	
Rotenone (10 μM)	1260	1995	180	1	32	139	59	102
				6	24	78	19	
				U	21	95	84	
—	1860	1143	459	1	263	288	379	92
				6	434	278	125	
				U	266	339	309	
Phenazine methosulphate (20 μM)	2610	392	1532	1	155	151	1157	79
				6	321	289	169	
				U	193	261	653	
—	1000	740	284	1	65	202	202	106
				6	151	222	97	
				U	87	253	206	
Rotenone (10 μM) and phenazine methosulphate (20 μM)	1770	470	1145	1	55	103	777	87
				6	136	229	112	
				U	74	217	441	

Table 2. *Influence of rotenone and phenazine methosulphate on the rates of fatty acid synthesis and pentose phosphate-cycle activity*

The values for the amount of glucose converted into fatty acids were calculated from the specific radioactivity of the [U- 14 C]glucose used. The contribution of the pentose phosphate cycle to glucose catabolism was calculated from 14 CO $_2$ liberation from [1- 14 C]- and [6- 14 C]-glucose as described by Katz *et al.* (1966).

Conditions	Metabolism of glucose (nmol/2h per 100 μ mol of triglyceride)	
	[U- 14 C]Glucose converted into fatty acids	Glucose catabolized via pentose phosphate cycle
—	147	62
Rotenone (10 μ M)	21	13
—	266	111
Phenazine metho- sulphate (20 μ M)	193	486
—	87	47
Rotenone (10 μ M) and phenazine metho- sulphate (20 μ M)	74	331

on the other hand, was only decreased to about 50%. Table 2 shows that both fatty acid synthesis and pentose phosphate-cycle activity are inhibited by rotenone (by about 85 and 80% respectively).

Phenazine methosulphate enhanced glucose uptake to a considerable extent (Table 1). The ratio of lactate to pyruvate production changed from 2.6 to 0.25, reflecting a drastic shift of the cytoplasmic NAD $^+$ /NADH couple to the oxidized state. In spite of the marked change of the redox state the incorporation of 14 C into fatty acids and glyceride glycerol from [U- 14 C]glucose was only slightly decreased. The amount of 14 CO $_2$ evolved from [1- 14 C]glucose was 3 times that in the controls and 14 CO $_2$ production from [U- 14 C]glucose was doubled. These results indicate that a marked stimulation of glucose oxidation via the pentose phosphate cycle and tricarboxylic acid cycle occurred under the influence of phenazine methosulphate, whereas fatty acid synthesis was only slightly affected (Table 2).

This stimulatory effect of phenazine methosulphate on the pentose phosphate cycle was more pronounced in cells from starved animals (Table 3). Although glucose metabolism via this pathway increased more than ten times the rate of fatty acid synthesis was almost unchanged under the influence of this agent. Glucose uptake was unchanged in these experiments and no accumulation of 6-phosphogluconate could be detected.

When both rotenone and phenazine methosulphate were present, a similar metabolic fate of glucose utilized was observed to that in the presence of phenazine methosulphate alone, suggesting that

the effect of rotenone was completely overcome by phenazine methosulphate.

The flow of glucose carbon via the pentose phosphate cycle was more than sixfold increased in the presence of both rotenone and phenazine methosulphate compared with the appropriate controls (Table 2). This fact provides strong evidence that rotenone exerts no direct effect on the pentose phosphate cycle.

Table 4 shows the effects of 6-aminonicotinamide *in vitro* on fatty acid synthesis, pentose phosphate-cycle activity and 6-phosphogluconate formation in isolated fat-cells under various conditions. In these experiments tissue and cells were kept in a glucose-free medium for about 2.5h. The relatively small amount of glucose utilized in these experiments, as well as the low rate of fatty acid synthesis and pentose phosphate-cycle activity in the controls, is probably due to this treatment.

Insulin treatment resulted, as expected, in a marked increase of glucose utilization, fatty acid synthesis and pentose phosphate-cycle activity. The 6-phosphogluconate content was not measurable with the enzymic assays used, in either the controls or the insulin-treated samples.

In cells preincubated with 6-aminonicotinamide the amount of glucose catabolized was slightly increased. Insulin, however, did not stimulate glucose catabolism to the same extent as had been observed without 6-aminonicotinamide. Similar results have been reported from rat adipose tissue by von Bruchhausen & Herken (1966). In spite of an accumulation of 6-phosphogluconate in cells preincubated with

Table 3. *Effect of phenazine methosulphate on glucose uptake and the rates of NADPH utilization for fatty acid synthesis and NADPH production via the pentose phosphate cycle in fat-cells from animals starved for 48 h*

The NADPH requirement for fatty acid synthesis and NADPH production in the pentose phosphate cycle were calculated as follows. For the reduction of 1 μmol of acetyl-CoA to an ethyl group, 2 μmol of NADPH are required. Assuming the mean chain length of the fatty acid to be 17 carbon atoms, 0.875 μmol of NADPH is required for 1 μg -atom of carbon of newly synthesized fatty acids. For each μmol of glucose metabolized via the pentose phosphate cycle 3 μmol of CO_2 and concomitantly 6 μmol of NADPH are produced.

Conditions	Metabolism (nmol/2h per 100 μmol of triglyceride)			
	Glucose catabolized	6-Phospho- gluconate content	NADPH required for fatty acid synthesis	NADPH produced in pentose phosphate cycle
48 h starved	1750	<1	50	157
48 h starved and phenazine metho- sulphate (20 μM)	1670	<1	76	2676

Table 4. *Effect of 6-aminonicotinamide in vitro on glucose uptake and 6-phosphogluconate content and on the rates of NADPH utilization (fatty acid synthesis) and production (pentose phosphate cycle) in isolated fat-cells*

For calculations see the legend to Table 3.

Conditions	Metabolism (nmol/2h per 100 μmol of triglyceride)			
	Glucose catabolized	6-Phospho- gluconate content	NADPH required for fatty acid synthesis	NADPH produced in pentose phosphate cycle
Control	562	<1	153	131
Insulin (1 unit)	1942	<1	1738	955
6-Aminonicotinamide	701	13	200	126
6-Aminonicotinamide and insulin (1 unit)	1080	73	635	492

6-aminonicotinamide, no appreciable changes in the relative rates of fatty acid synthesis and pentose phosphate pathway were found in the absence or presence of insulin.

Discussion

Rotenone is known to inhibit intramitochondrial electron transport. In our experiments with adipocytes all decarboxylation sites of glucose breakdown beyond the C_3 -compound level have been found to be blocked in the presence of rotenone. This inhibition can be attributed to an accumulation of intramitochondrial NADH. Consequently, presumably because of the lack of acetyl-CoA, fatty acid

synthesis was decreased by 85%; at the same time pentose phosphate-cycle activity was diminished to the same extent. The increase in the ratio of lactate to pyruvate production in the presence of rotenone reflects an increase of the NADH/NAD⁺ ratio in the cytosol.

It should be noted that in the presence of rotenone a similar metabolic pattern is observed to that in cells from starved animals, where most of the glucose catabolized was found to be converted into C_3 compounds, mainly lactate (Table 1 of Kather *et al.*, 1972). These similar results support the suggestion of Del Boca & Flatt (1969) that the impairment of lipogenesis in tissue from starved animals is due to the decreased availability of acetyl-CoA.

From these results it cannot be concluded whether rotenone affects directly both lipogenesis and pentose phosphate-cycle activity, or whether the decreased rate of the pentose phosphate pathway is a consequence of decreased fatty acid synthesis. In ascites-tumour cells it has been found that the pentose phosphate cycle is not influenced by rotenone in spite of a complete block of biosynthetic pathways (K. Brand, O. Dionisi & T. Terranova, unpublished work).

Phenazine methosulphate is known to stimulate the pentose phosphate cycle by accepting electrons and thus regenerating NAD^+ and NADP^+ (Gumaa & McLean, 1969; Katz & Wals, 1970; Saggerson & Greenbaum, 1970; Greenbaum *et al.*, 1971; Katz & Wals, 1971).

In our experiments the presence of phenazine methosulphate caused about a fivefold (cells from control animals) to tenfold (cells from starved animals) stimulation of glucose catabolism via the pentose phosphate cycle, and an about twofold increase in tricarboxylic acid-cycle activity in cells from control animals. These results are in agreement with those reported by Saggerson & Greenbaum (1970) and Katz & Wals (1971) in adipose tissue, and by Greenbaum *et al.* (1971) in liver. They indicate that none of the enzymes of the pentose phosphate cycle and the tricarboxylic acid cycle operates normally at maximal capacities. Phenazine methosulphate had little effect on lipogenesis in our experiments, whereas the redox state of the cells was markedly altered under the influence of this dye (the lactate to pyruvate ratio was changed from 2.6 to 0.25). These results are similar to the findings of Katz & Wals (1971) in adipose tissue. They concluded that the redox state of the cells does not control lipogenesis.

Addition of rotenone to the samples containing phenazine methosulphate did not alter the effect of the latter (Tables 1 and 2). This dye obviously is able to overcome the metabolic block caused by rotenone because of its ability to transfer reducing equivalents from NADH to the dissolved oxygen in the medium. From this result it can be concluded that rotenone has no direct influence on either lipogenesis or the pentose phosphate pathway.

The decrease in fatty acid synthesis in the presence of rotenone can therefore be attributed to a decrease in pyruvate decarboxylation, which consequently leads to a decreased availability of acetyl-CoA. Since a direct effect of rotenone on the pentose phosphate cycle has been excluded the decreased rate of this pathway in the presence of rotenone has to be considered as a consequence of decreased lipogenesis.

The rate of glucose catabolism via the pentose phosphate cycle in adipocytes therefore appears to be completely determined by the requirement of NADPH for fatty acid synthesis. This strong

dependence supports the concept of a mass-action control of this pathway in fat-cells with respect to the availability of NADP^+ , as reported by Brand *et al.* (1970) in human erythrocytes.

To localize the site of mass-action control in this pathway, experiments with 6-aminonicotinamide were carried out (Table 4). 6-Aminonicotinamide was reported to be incorporated into cellular nicotinamide nucleotides yielding the corresponding 6-aminonicotinamide analogues of NAD and NADP (Johnson & McColl, 1956).

The resulting 6-aminonicotinamide nucleotides were found to act neither as acceptors of reducing equivalents nor as donors (Brunnemann *et al.*, 1964). A strong inhibitory effect on 6-phosphogluconate dehydrogenase after application of 6-aminonicotinamide *in vivo* was demonstrated in rat brain (Herken *et al.*, 1969; Lange *et al.*, 1970), kidney (Lange & Proft, 1970) and in rat embryos (Köhler *et al.*, 1969).

The accumulation of 6-phosphogluconate observed after treatment with 6-aminonicotinamide indicates that 6-phosphogluconate dehydrogenase is inhibited. In spite of this inhibition the flux of glucose carbon via the oxidative pentose phosphate pathway and the rate of fatty acid synthesis were not greatly affected. Further stimulation, but not to the same extent as in the absence of 6-aminonicotinamide, could be obtained by the addition of insulin, accompanied by an increased accumulation of 6-phosphogluconate (Table 4).

From these results we conclude the following: (1) An accumulation of 6-phosphogluconate does not necessarily indicate a decreased flux of glucose molecules through the oxidative branch of the pentose phosphate pathway, as assumed by Herken *et al.* (1969) (Table 4). (2) 6-Phosphogluconate dehydrogenase, even when inhibited by 6-aminonicotinamide, is not the rate-limiting enzyme of this pathway in non-stimulated metabolism (Table 4). (3) Pentose phosphate-cycle activity appears to be controlled by the rate of regeneration of NADP^+ at the site of glucose 6-phosphate dehydrogenase, since even in cells from starved animals, in which pentose phosphate-cycle activity was extremely low but capable of stimulation by phenazine methosulphate, no accumulation of 6-phosphogluconate was observed (Table 3). This conclusion is in agreement with the proposal of Greenbaum *et al.* (1971) from studies in rat liver.

References

- Brand, K., Arese, P. & Rivera, M. (1970) *Hoppe-Seyler's Z. Physiol. Chem.* **351**, 501-508
- Brin, M. & Yonemoto, R. H. (1958) *J. Biol. Chem.* **230**, 307-317
- Brunnemann, A., Coper, H. & Neubert, D. (1964) *Naunyn-Schmiedeberg's Arch. Exp. Pathol. Pharmacol.* **246**, 437-451

- Del Boca, J. & Flatt, J. P. (1969) *Eur. J. Biochem.* **11**, 127-134
- Greenbaum, A. L., Gumaa, K. A. & McLean, P. (1971) *Arch. Biochem. Biophys.* **143**, 617-663
- Gumaa, K. A. & McLean, P. (1969) *Biochem. J.* **115**, 1009-1029
- Herken, H., Lange, K. & Kolbe, H. (1969) *Biochem. Biophys. Res. Commun.* **36**, 93-100
- Holzer, H. & Witt, I. (1958) *Angew. Chem.* **70**, 439
- Johnson, W. J. & McColl, J. P. (1956) *Fed. Proc. Fed. Amer. Soc. Exp. Biol.* **15**, 284
- Kather, H., Rivera, M. & Brand, K. (1972) *Biochem. J.* **128**, 1089-1096
- Katz, J. & Wals, P. A. (1970) *J. Biol. Chem.* **245**, 2546-2548
- Katz, J. & Wals, P. A. (1971) *Arch. Biochem. Biophys.* **147**, 405-418
- Katz, J., Landau, B. R. & Bartsch, G. E. (1966) *J. Biol. Chem.* **241**, 727-740
- Köhler, E., Barrach, H.-J. & Neubert, D. (1969) *FEBS Lett.* **6**, 225-228
- Krebs, H. A. & Henseleit, K. (1932) *Hoppe-Seyler's Z. Physiol. Chem.* **210**, 33-66
- Lange, K. & Proft, E. R. (1970) *Naunyn-Schmiedeberg's Arch. Pharmacol. Exp. Pathol.* **267**, 177-180
- Lange, K., Kolbe, H., Keller, K. & Herken, H. (1970) *Hoppe-Seyler's Z. Physiol. Chem.* **351**, 1241-1252
- Roigas, H., Zoellner, E., Jacobasch, G., Schultze, M. & Rapoport, S. (1970) *Eur. J. Biochem.* **12**, 24-30
- Saggerson, E. D. & Greenbaum, A. L. (1970) *Biochem. J.* **119**, 193-220
- von Bruchhausen, F. & Herken, H. (1966) *Naunyn-Schmiedeberg's Arch. Pharmacol. Exp. Pathol.* **254**, 388-400