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Effect of Phenformin on Gluconeogenesis in Perfused Rat Liver. II. Dependence on the ATP/ADP Ratio

J. M.ª Medina,* F. Sánchez-Medina and F. Mayor

Departamento de Bioquímica
Universidad de Granada
Granada (Spain)

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The effect of phenformin (phenethylbiguanide) on gluconeogenesis from lactate, pyruvate, oxalacetate, glycerol, dihydroxyacetone, fructose and mannose in perfused rat liver was studied. 1 mM phenformin inhibited gluconeogenesis from all these substrates. The time-course of the inhibition of gluconeogenesis from lactate showed a good correlation between lactate removal and glucose production, indicating that the effect of phenformin on gluconeogenesis was independent on the activity of the mixed function oxidation system, in perfused rat liver.

Our findings suggest an indirect inhibition of phenformin on gluconeogenesis likely related with its effect on oxidative phosphorylation. The fall in ATP/ADP ratio and the increase in total ketone bodies in liver following perfusion with phenformin seem to support this suggestion. The well-known effect of ethionine in decreasing hepatic ATP levels and gluconeogenesis *in vivo* prompted us to investigate its influence in perfused rat liver. The inhibition of the rate of glucose production in these conditions after ethionine administration was very similar to that found perfusing with phenformin.

In order to explain the hypoglycaemic effect of biguanides, several mechanisms of action have been proposed. STEINER and WILLIAMS (30, 31) suggested that biguanides produce a partial cellular anoxia, which leads to an increased perypheral uptake of glucose and anaerobic glycolysis. SCHÄFER (26, 27) demonstrated that these compounds inhibited the mitochondria energy-transducing reaction. On the other hand, LOUBATIÈRES (19) found an increase of insulin realease by biguanides in dog pancreas. An inhibition of intestinal glucose absortion has also been proposed by BIRO *et al.* (4). The inhibition of ATP synthesis produced as a consequence of the action of biguanides on cell respiration has beed invoked by CZYZIC (5) to explain this effect. Finally, a specific inhibition of gluconeogenesis has been postulated by MEYER (21) in rat kidney cortex.

The inhibition of gluconeogenesis by phenformin (phenethylbiguanide) in guinea-pig liver was demonstrated by ALTS-

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CHULD and KRUGER (3) and HAECKEL and HAECKEL (10). However, according to ALTSCHULD and KRUGER, phenformin has little effect on rat liver. The results reported in the first part of this work (20) showed a clear inhibition of rat liver gluconeogenesis from lactate, glycerol and fructose by phenformin probably related to the inhibitory effect of this drug on the oxidative phosporylation.

In addition to its pharmacological significance, the inhibitory effect of phenformin on gluconeogenesis might be utilized as a tool in the study of glucose metabolism. Therefore, in the present work we tried to evaluate further the mechanism of action of phenformin on rat liver gluconeogenesis. We have studied its effect using several substrates and determined the liver energetic and redox states following perfusion with phenformin. Moreover, the inhibitory effect on the rate of gluconeogenesis produced by ethionine, a substance that leads to a decrease in the ATP level, has been also investigated.

Materials and Methods

Chemicals. — D-L Ethionine and glucose oxidase where obtained from Sigma Chemical Co. (USA). All the other enzymes used were purchased from Boehringer Manheim (Germany). Bovine serum albumin powder fraction V was obtained from Armour Pharmaceutical Co. Ltd. Eastbourne, Sussex (England). Standard analytical grade laboratory reagents and coenzymes were obtained from Sigma, Merck (Darmstadt) and Boehringer. Sodium pentobarbital was a gift from Laboratorios Abbot, Madrid. Phenformin was kindly supplied by Laboratorios Funk, Manlleu, Barcelona.

Treatment of the Animals. — Fasted (48h) female Wistar rats weighing 150-200 g were used in all experiments. For *in vivo* experiments, the rats were intraperitoneally injected with a single dose (100 mg/kg body weight) of phenformin in saline solution two hours before they were sacrified. In the experiments with ethionine, the rats were i.p. injected with D-L ethionine (750 mg/kg body weight in saline solution) 10 hours before perfusion. Control rats were injected with saline solution.

Perfusion Method. — The perfusion procedure, based on the methods of MILLER *et al.* (22) and SCHIMASSEK (25) has been described by HEMS *et al.* (12). The perfusate consisted of Krebs-Henseleit physiological saline (15), bovine serum albumin and washed human red blood cells, stored 30 days at 4° C in citrate-dextrose anticoagulant solution. Other experimental details have been previously given (20).

Tissue Treatment. — For *in vivo* experiments, the rats were killed by cervical dislocation (with minimal stress to the animal). A portion of liver was rapidly excised and clamped between metal tongs previously cooled in liquid nitrogen (38). The time elapsing between dislocation of the neck and deep-freezing the liver was 8-10 sec. The frozen liver was pulverized in a mortar, extracted with perchloric acid solution and neutralized with KOH, as described by WILLIAMSON *et al.* (37). The liver extracts were shaken with Florisil (0.1 g/ml) and centrifuged off. The supernatant fluid was used for the analysis. This treatment removed flavines from the solution and decreased the slow non-enzymic oxidation of NADH observed with untreated samples. However, as florisil treatment interfered with the recovery of the adenine nucleotides and inorganic phosphate, supernatant fluid before florisil treatment was used for their determination. The same treatment was applied to perfused livers, the tissue samples being taken after 90 min. of perfusion with substrate.

Analytical Methods. — Glucose was determined by the glucose oxidase method, according to KREBS *et al.* (16, 17) Lactate and pyruvate were determined by the method of HOHORST *et al.* (13), β-hydroxybutyrate and acetoacetate as described by WILLIAMSON *et al.* (36), ATP by the method of LAMPRECH and TRAUTSCHOLD (18), ADP and AMP by the method of ADAM (1) and inorganic phosphate according to FISKE and SUBBAROW (9). Haemoglobin was measured as cyanmethaemoglobin according to EVELYN and MALLOY (7).

Results and Discussion

We have investigated the effect of 1 mM phenformin on gluconeogenesis from lactate, pyruvate, oxalacetate, glycerol, dihydroxyacetone, fructose and mannose in perfused rat liver. In all cases, a clear inhibition was found, confirming our preliminary results using lactate, glycerol and fructose as gluconeogenic precursors (20). The inhibitory effect was higher when lactate (72 %), pyruvate (90 %), glycerol (95 %) and dihydroxyacetone (79 %) were used. The effect on gluconeogenesis from oxalacetate, fructose, and mannose was less pronounced (51 %, 44 % and 34 % respectively) (fig. 1).

Gluconeogenesis from lactate and glycerol was not inhibited when the concentration of phenformin was 0.1 mM. Still, an increase in glucose production was observed. This paradoxical effect may be explained by the fact that phenformin accelerates the flux of the perfusion me-

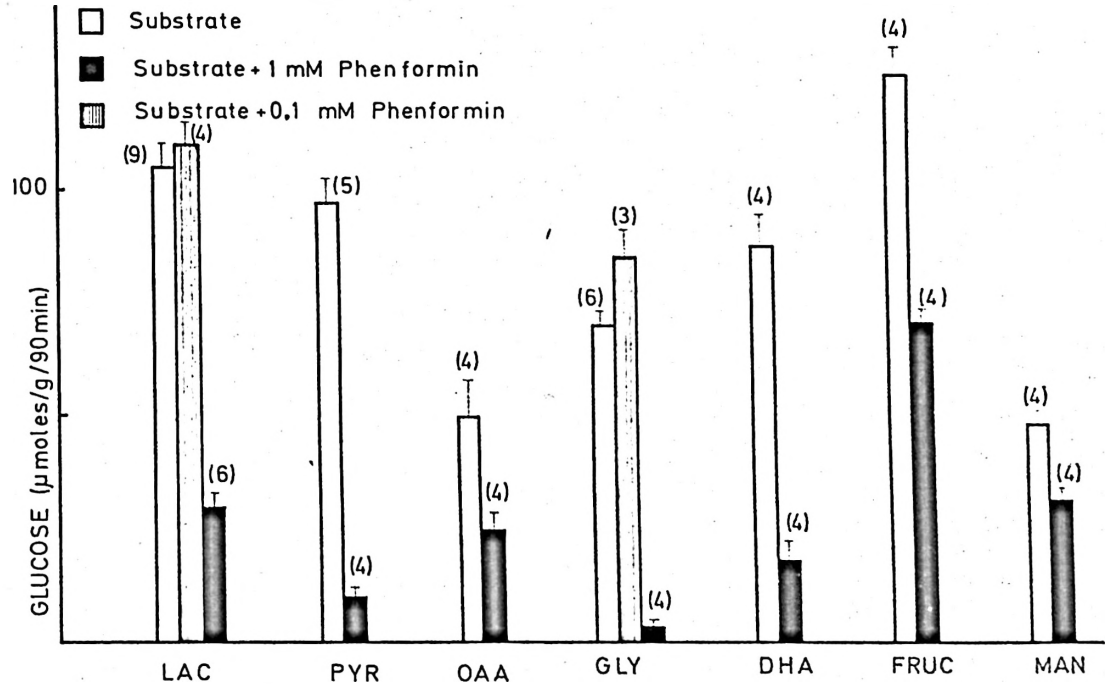

Fig. 1. *Effect of phenformin on gluconeogenesis in perfused rat liver.*
All experiments were carried out with 10 mM substrate. Rats were starved for 48 h. Glucose values are given in μmoles per g ol liver wet weight produced in 90 min. of perfusion with substrate. Vertical bars indicate ± S.E.M. with the number of observations in parenthesis. LAC, means lactate; PYR, pyruvate; OAA. oxalacetate; GLY, glycerol; DHA, dihidroxyacetone; FRUC, fructose and MAN, mannose.

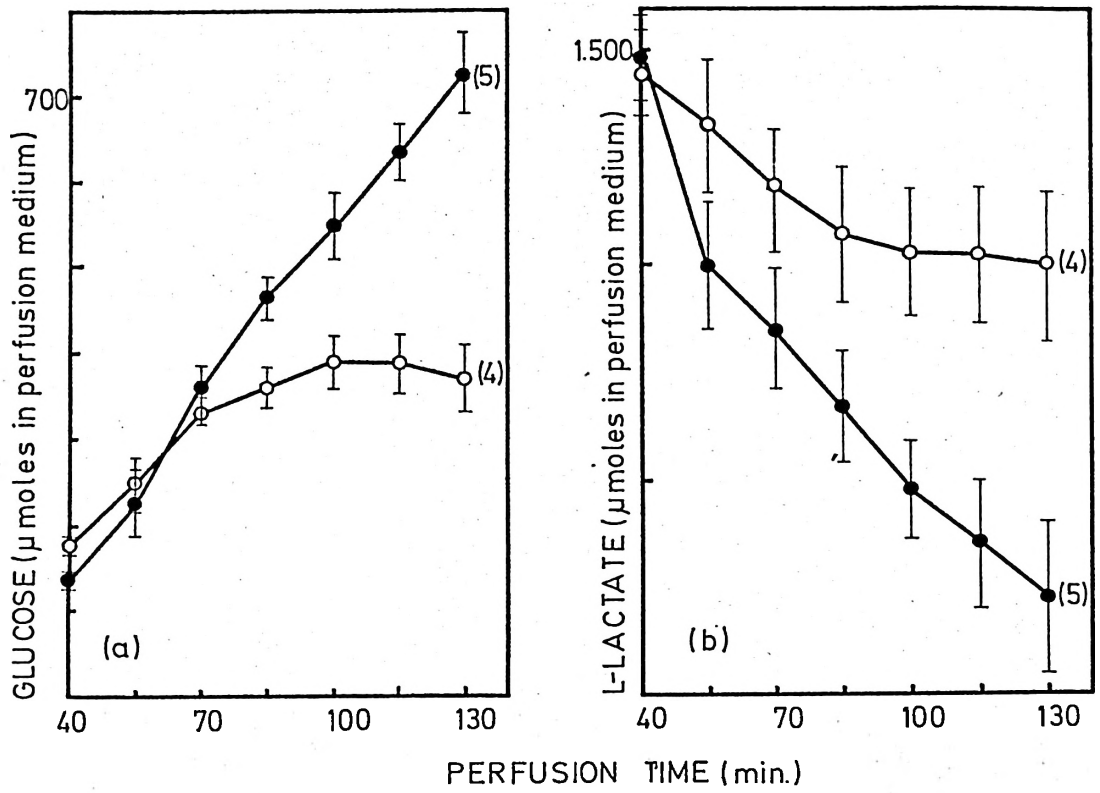

Fig. 2. *Time-course of the inhibition of gluconeogenesis from lactate by phenformin.* (●), 10 mM lactate; (○), 10 mM lactate + 1 mM phenformin. (a) Glucose found in the perfusion medium. (b) Lactate found in the perfusion medium. Substrate and inhibitor were added at 38 min. Rats were starved for 48 h. Vertical bars indicate ± S.E.M. Number of observations in parenthesis.

dium through the liver, increasing the metabolic activity of the tissue.

The time-course inhibition of gluconeogenesis from lactate by 1 mM phenformin is shown in the fig. 2. Assuming that two molecules of lactate are converted into one molecule of glucose, the stoichiometry of the reaction is maintened in all cases along the time of perfusion. As it can be seen, no removal of lactate is observed during the inhibition of glucose production by phenformin.

THURMAN and SCHOLZ (34) have stated the problem of whether or not the metabolism of biguanides by the mixed function oxidation system of liver is related to their ability to inhibit gluconeogenesis. In this case, some glucose-6 phosphate is diverted to the pentose phosphate shunt in order to produce reduced NADP and more than two molecules of lactate are removed per molecule of glucose produced. The good correlation found in our experiments between glucose production and lactate removal precludes such a mechanism to explain the inhibition of rat liver gluconeogenesis by phenformin.

As figure 2 shown, 30 min. were needed before 1 mM phenformin developed a clear inhibitory effect. As discussed earlier (20), this lag period suggests that phenformin might accumulate in liver until an

inhibitory concentration is reached or, alternatively, that there is an indirect effect produced by the inhibition of another metabolic process, whose end-product is needed for gluconeogenesis to take place (i.e. ATP). Another explanation would be that the effect of phenformin is actually achieved by one of its metabolites. However, HAECKEL (11) has reported that the main metabolite of phenethylbiguanide i.e. p-oxiphenethylbiguanide is unable to inhibit gluconoegenesis in perfused guinea pig liver.

The lag period was no longer found when phenformin was added to the medium 30 min. before the substrate (fig. 3). In these conditions, 1 mM phenformin completely suppressed the production of glucose and even 0.1 mM phenformin showed a clear inhibitory effect (44 %).

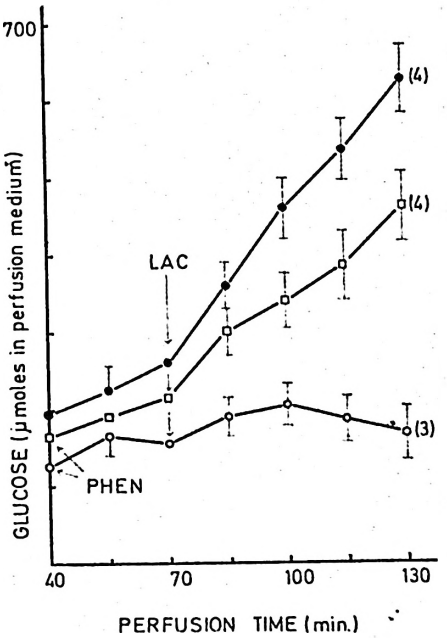

Fig. 3. *Inhibition of gluconeogenesis from lactate by phenformin added before substrate.* Glucose found in the perfusion medium using 10 mM lactate, (●); 10 mM lactate + 1 mM phenformin, (○); 10 mM lactate+0.1 mM phenformin, (□); inhibitor was added at 38 min. and substrate at 68 min. Rats were starved for 48 h. Vertical bars indicate ± S.E.M. Number of observations in parenthesis. LAC means lactate and PHEN, phenformin.

Several mechanisms of gluconeogenesis inhibition by phenformin have been proposed: inhibition of pyruvate oxidation (10, 14), decreased flux through the Krebs cycle (14), inhibition of conversion of pyruvate to malate (10) and the conversion of 3-phosphoglycerate to 3-phosphoglyceraldehyde (10, 35). Our results using glycerol, dihydroxyacetone, fructose and mannose (fig. 2) point out that the gluconeogenic pathway is also inhibited above the 3-phosphoglyceraldehyde dehydrogenase step. This finding is consistent with the results of ALLEYNE *et al.* (2) who showed that phenformin inhibits in rat renal gluconeogenesis from glutamine, glutamate, succinate, oxalacetate, and fructose.

These results seem to suggest a direct inhibition of fructose-1,6-diphosphatase and/or glucose-6-phosphatase. However, PATRICK (23) found no effect *in vitro* of phenformin on these enzymes in rat kidney, being this tissue more sensitive to phenformin than the liver is. In addition, we have found a clear inhibition of glucose synthesis from mannose, a substrate that enters the gluconeogenic pahtway above the step catalyzed by fructose-1,6-diphosphatase. On the other hand, the activity of glucose-6-phosphatase was unaffected after intraperitoneal injection of phenformin (100 mg/kg body weight) (Medina, J. M. Unpublished results).

These observations strongly suggest an indirect inhibitory effect of phenformin on gluconeogenesis. Since the inhibition of oxidative phosphorylation by biguanides is well established (26, 27) a fall in the ATP level and/or a change in the cellular redox state could lead to a decrease in gluconeogenesis. The data of Table I show that the *in vivo* treatment with phenformin did not produce any changes in the lactate/pyruvate and β-hydroxybutyrate/acetoacetate ratios, indicating that the redox state expressed by these ratios remains unchanged.

Table I. *Concentration of some substrates of NAD-linked dehydrogenase systems in livers of starved (48 h.) rats treated with phenformin (100 mg/kg).*

The concentrations of the metabolites are expressed as μmoles/g liver wet weight. The values are means ± S.E.M. Number of observations, for each group, 4. For experimental details see the text.

State of animals	Lactate	Pyruvate	Acetoacetate	β-Hydroxybutyrate	[Lactate]/[Pyruvate]	[β-Hydroxybutyrate]/[Acetoacetate]	Total Ketone bodies
Untreated	0.48±0.07	0.021±0.002	0.59±0.02	1.65±0.06	22.8±0.1	2.8±0.3	2.24±0.09
Phenformin-treated	0.58±9.09	0.029±0.006	0.58±0.05	1.43±0.05	20.0±0.2	2.5±0.4	2.02±0.08

Furthermore, no change occured in the β-hydroxybutyrate/acetoacetate ratio in the livers perfused with phenformin (Table II). The levels of lactate and pyruvate were not determined in these conditions since the experiments were carried out using lactate as gluconeogenic substrate.

Table III shows that no significative differences were observed in the ATP/ADP ratios in the liver of rats injected with phenformin ($P < 0.6$). However, perfusion with phenformin produced a clear decrease in the ATP/ADP ratio whereas the AMP level increased (Table IV). Because of the energy requeriments of glucose synthesis, the fall in the ATP/ADP ratio may account for the observed inhibition of gluconeogenesis. On the other hand, the increase in AMP, a powerful inhibitor of fructose 1,6-diphosphatase (32) might be taken into account. In this regard, ALTSHULD and KRUGER have pointed out that the increase in the level of AMP may explain the inhibition of gluconeogenesis by phenformin in guinea pig liver (3).

The lack of effect of phenformin on hepatic ATP and AMP levels *in vivo* (Table III) may be explained by the fact that phenformin is highly metabolized in rat liver (11). On the contrary, as stated above, the mixed function oxidation system is not operative in perfused rat liver. Likewise, the amount of total ketone bodies was increased by phenformin only in perfused rat liver (Tables III and IV). The decreased flux of the tricarboxilyc acid cycle (35) produced by the biguanides can account for this finding.

Another fact that can contribute to explain the effect of phenformin on gluconeogenesis, is the well established decrease in the citrate concentration produced by

Table II. *Concentrations of ketone bodies in livers of starved (48 h.) rats perfused with phenformin.*

Livers were perfused with standard medium being substrate and inhibitor added at 38 min. Liver samples were taken at 130 min. The concentrations of ketone bodies are expressed as μmoles/g liver wet weight. The values are means ± S.E.M. Number of observations, for each group, 4. For experimental details see the text.

Perfusion conditions	β-hidroxybutyrate	Acetoacetate	Total ketone bodies	β-hidroxybutyrate/acetoacetate
10 mM lactate	0.422 ± 0.061	0.131 ± 0.013	0.553 ± 0.057	3.40 ± 0.665
10 mM lactate + 1 mM phenformin	0.706 ± 0.042	0.194 ± 0.044	0.900 ± 0.071	3.88 ± 0.581

Table III. *Concentrations of adenin-nucleotides in livers of starved (48 h.) rats treated with phenformin (100 mg/kg).*

The concentrations of the nucleotides are expressed as μmoles/g liver wet weight. The values are means ± S.E.M. Number of observations, for each group, 4. For experimental details see the text.

State of animals	ATP	ADP	AMP	Inorganic Phosphate	Total nucleotides	ATP/ADP
Untreated	1.61±0.08	1.29±0.07	0.45±0.01	4.87±0.04	3.34±0.07	1.24±0.08
Phenformin-treated	2.02±0.08	1.78±0.13	0.50±0.02	5.05±0.17	4.31±0.32	1.13±0.11

Table IV. *Concentrations of adenin-nucleotides in livers of starved (48 h.) rats perfused with phenformin.*

Livers were perfused with standard medium being the substrate and inhibitor added at 38 min. Liver samples were taken at 130 min. The concentrations of nucleotides are expressed as μmoles/g liver wet weight. The values are means ± S.E.M. Number of observations, for each group, 4. For experimental details see the text.

Perfusion conditions	ATP	ADP	AMP	Inorganic Phosphate	Total nucleotides	ATP/ADP
10 mM lactate	1.80±0.20	0.89±0.17	0.39±0.09	4.15±0.72	3.08±0.44	2.15±0.24
10 mM lactate + 1 mM phenformin	0.87±0.03	0.94±0.07	0.59±0.08	6.65±0.41	2.41±0.08	0.92±0.04

this compound (10, 35) which coud lead to the deinhibition of phosphofructokinase (24). As pointed out by DAVIDOFF (6) «a fairly small decrease in citrate production and/or release from mitochondrial rather than a drop in ATP levels due to limited respiration could represent the effect of phenethylbiguanide which was primarily responsible for the drug-induced increase in rate of peripheral glycolysis in the fasting state». However, we have found that the addition of 1 mM citrate to the perfusion medium was unable to counteract the inhibitory effect of 1 mM phenformin on gluconeogenesis (glucose production was 27.3±2.7 (4) μmoles/g/90 min.) In any case, such a mechanism could not easily explain the inhibition found using mannose as substrate.

Ethionine is known to decrease the concentration of ATP in the liver by the formation of S-adenosylethionine at high rate (8, 29). Table V shows the results obtained when starved rats treated with ethionine were perfused with 10 mM lactate. As expected, gluconeogenesis was decreased, the level of adenin nucleotides and the rate of glucose production being very similar to those obtained perfusing with 1 mM phenformin and 10 mM lactate (Fig. 1 and Table IV).

In this connection, it must be pointed aut that TANI and OGATA (33) have reported the inhibition of gluconeogenesis following ethionine administration in experiments carried out *in vivo*. The suppression of gluconeogenesis was parallel with the decrease of the hepatic ATP content. On the other hand, in livers from fasted guinea pig, ALTSHULD and KRUGER (3) have found a correlation between the percent inhibition of gluconeogenesis produced by phenformin and the decrease in the ATP liver concentration.

In the perfused rat liver is very difficult to establish wheter or not the drop in the

Table V. *Rate of gluconeogenesis and concentrations of adenin-nucleotides in livers of starved (48 h.) rats treated with ethionine and perfused with 10 mM lactate.*
The rats were injected with D-L ethionine (750 mg/kg body weight in saline solution) 10 hours before perfusion. Livers were perfused with standard medium and the substrate (10 mM L-lactate) was added after 38 minutes. Liver samples were taken at 130 minutes. The rate of gluconeogenesis is given in μmoles of glucose produced in 90 min./g of liver wet weight. The concentrations of nucleotides are expressed in μmoles/g of liver wet weight. The values are means ± S.E.M. Number of observations, for each group, 6. For experimental details see the text.

Rate of gluconeogenesis	Concentrations of adenin-nucleotides			
	ATP	ADP	AMP	ATP/ADP
0.39 ± 0.10	0.83 ± 0.11	0.84 ± 0.11	0.39 ± 0.09	1.11 ± 0.22

ATP/ADP ratio produced by phenformin is primary responsible for the depressed gluconeogenesis rate. In any case, the effect of biguanides at high concentration *in vitro* has not physiological significance and the problem seems not easy to resolve because of experimental difficulties to measure the mitochondrial ATP pool. Biguanides act at mitochondrial level and, as indicated by SHÄFER (28), there might well be some undetectable change of ATP concentration in the mitochondrial compartment if low concentration of biguanides are used.

Resumen

Se ha estudiado el efecto de la fenformina (fenetilbiguanida) sobre la gluconeogénesis a partir de lactato, piruvato, oxalacetato, glicerol, dihidroxiacetona, fructosa y manosa en hígado perfundido de rata. La fenformina, a concentración 1 mM, inhibe la gluconeogénesis a partir de todos estos precursores. Existe una buena correlación entre el consumo de lactato y la producción de glucosa a lo largo del tiempo de perfusión, lo que indica que el efecto de la fenformina sobre la gluconeogénesis es independiente de la actividad de los sistemas oxidantes de función mixta en hígado perfundido de rata.

Nuestros resultados sugieren que la fenformina inhibe la gluconeogénesis de manera indirecta, muy probablemente en relación con su efecto sobre la fosforilación oxidativa. El decrecimiento en la razón ATP/ADP y el incremento en la concentración de los cuerpos cetónicos totales en hígado tras la perfusión con fenformina parecen apoyar dicha sugerencia. El bien conocido efecto depresor de la etionina sobre los niveles hepáticos de ATP y la gluconeogénesis *in vivo* nos indujo a investigar su influencia en hígado perfundido. La inhibición de la velocidad de la producción de glucosa en estas condiciones tras la administración de etionina fue muy similar a la encontrada perfundiendo con fenformina.

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