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INHIBITION OF GLUCONEOGENESIS IN ISOLATED PERFUSED RAT LIVER BY CLANOBUTIN

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Abstract—The effect of clanobutin {4-[*p*-chloro-*N*-(*p*-methoxyphenyl-)benzamido]butyric acid} on gluconeogenesis from lactate + pyruvate (1.6 + 0.2 mmoles/l) as precursors in isolated perfused liver of fasted rats was investigated. Glucose production was dose dependent inhibited up to a maximum of 81 ± 3 per cent; the half-maximum concentration of clanobutin was 0.33 ± 0.04 mmoles/l. Buformin and phenformin, respectively, used as references showed no effect on gluconeogenesis under the given experimental conditions. Oxygen uptake was not inhibited by clanobutin at concentrations up to 0.14 mmoles/l. At higher concentrations, the inhibitory effect was smaller than observed for comparable buformin doses. According to our results, clanobutin appears to be a more potent and probably more specific inhibitor of gluconeogenesis than the therapeutically used biguanides—at least in the isolated perfused rat liver.

Numerous substances have been reported to inhibit gluconeogenesis in mammalian liver [1–14] (for a review see ref. 15). Various sites of inhibition have been discussed. Most inhibitory agents are supposed to act indirectly, e.g. via redox changes [16, 17], ATP generation [18, 19], futile cycles [11] etc., rather than directly by affecting a specific enzyme in the gluconeogenic sequence [4, 16]. Of these substances, only biguanides have been used in the past as therapeutic agents in the treatment of diabetes.

In the present communication, the effect of clanobutin§ {4-[*p*-chloro-*N*-(*p*-methoxyphenyl-)benzamido]butyric acid}—a recently synthesized compound, therapeutically employed mainly for its stimulating effect on bile and pancreas secretion, and antiulcerogenic properties [20, 21]—on gluconeogenesis in isolated perfused rat liver is reported. Clanobutin inhibits gluconeogenesis from lactate and pyruvate. Its effect is compared with that of biguanides.

MATERIALS AND METHODS

Liver perfusion. Male Sprague–Dawley rats SIV/50, weighing 170 ± 20 g, were fasted 20–22 hr prior to the perfusion experiments. The isolated livers were perfused in a non-recirculating system according to the technique previously reported by Scholz *et al.* [11]. The perfusion fluid was Krebs–Henseleit bicarbonate buffer [22], pH 7.4 saturated with an oxygen/carbon dioxide mixture (95/5), containing L-lactate and pyruvate (1.6 and 0.2 mmoles/l).

The fluid was pumped through a membrane oxygenator [11] prior to entering the liver via a cannula inserted in the portal vein. The perfusion effluent was collected via a cannula inserted in the vena cava and then passed an oxygen electrode before it was discharged. The livers were perfused for 2 hr. Clanobutin and biguanides (i.e. buformin or phenformin) were infused from the 32nd to the 64th minute of perfusion.

Analytical methods. Samples of the effluent were collected at 1-min intervals and analysed for glucose, lactate and pyruvate by standard enzymatic procedures.

The oxygen concentration in the effluent was monitored continuously, using a platinum electrode (Radiometer, Copenhagen). Metabolic rates (i.e. oxygen uptake, glucose production, lactate + pyruvate uptake) calculated from arteriovenous concentration differences and the constant flow rate of the perfusate, were expressed as micromoles per hour and gram wet weight of the liver.

Materials. Clanobutin, 4-[*p*-chloro-*N*-(*p*-methoxyphenyl-)benzamido]butyric acid, was available from our laboratories; buformin (butylbiguanide hydrochloride) was obtained from Dr. Brunnengräber, Chemische Fabrik Co. mbH, Lübeck; sodium L(+)-lactate was purchased from K. Roth OHG, Karlsruhe. Enzymes and pyruvate were supplied by Boehringer GmbH, Mannheim. All other chemicals were reagent grade from Merck, Darmstadt.

Statistical methods. For each experiment and parameter measured, observed values at the 28th, 48th and 80th minutes of perfusion were obtained as averages of five neighbouring values. For the parameters investigated, control experiments showed a slight linear decline in the course of the experiment. Therefore, the value expected without treatment at

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the 48th minute was calculated from the observed values at the 28th and 80th minutes by linear interpolation. Absolute and relative changes refer to the respective differences between observed and expected values at the 48th minute.

In order to establish dose–response relationships, a consistent model had to be fitted to all data. To this end, certain linear and non-linear functions were fitted to the data and their residual sums of squares were compared by means of the *F*-criterion. Standard errors and 95 per cent confidence limits were obtained for all model parameters.

RESULTS

Effects of clanobutin on glucose production, oxygen uptake and lactate + pyruvate uptake in perfused livers from fasted rats. In five control experiments (without substance) glucose production, oxygen uptake and lactate + pyruvate uptake showed a slight linear decline during the perfusion experiment due to increasingly impaired liver function. The deviation of the observed decline from strict linearity was less than 5 per cent for all parameters.

In contrast, the application of clanobutin from the 32nd to the 64th minute results in a prompt and pronounced inhibition of the above parameters. A typical experiment with clanobutin is shown in Fig. 1.

After 30 min of perfusion, the production of glucose and the uptake of oxygen reached steady-state rates around 50 and 150 μmoles/hr/g, respectively. Gluconeogenic precursors (lactate + pyruvate) were consumed at rates around 110 μmoles/hr/g. Infusion of clanobutin (0.7 mmoles/l) caused a rapid decrease in oxygen uptake and glucose production, paralleled by a diminished uptake of lactate + pyruvate. This experiment demonstrates that clanobutin inhibits gluconeogenesis by more than 60 per cent. The changes were rapidly reversed upon termination of clanobutin infusion.

Inhibition of gluconeogenesis as a function of clanobutin concentration. The dependence of inhibitory effect on clanobutin concentration is shown in Fig. 2. Inhibition of glucose production reached near-maximum values at clanobutin concentrations of 1.0 mmoles/l. Maximum effect was 81 ± 3 per cent inhibition; half-maximum effect was observed at 0.33 ± 0.04 mmoles/l.

Relationship between changes in glucose production, precursor and oxygen uptake. In Fig. 3, the changes of glucose production are plotted against changes in lactate + pyruvate uptake (Fig. 3A) and changes in oxygen uptake (Fig. 3B). The data clearly indicate a positive linear correlation between these parameters. The stoichiometry of precursors utilized and glucose formed was 1.9 : 1 on a molar basis and, therefore, was close to the theoretical value. The corresponding stoichiometry between oxygen utilized and glucose formed was 0.9 : 1. A value of 1 : 1 is expected on the basis of 6 moles of ATP consumed in the synthesis per 1 mole of glucose, on assumption of a mitochondrial P/O value of 3. A regression line with an intercept of 12 μmoles oxygen/hr/g provided the best fit to the data.

Comparison of effects of clanobutin and biguanides. In another series of experiments, the effects of buformin and phenformin were studied (Table 1). The concentrations used were approximately 30 times higher than the plasma concentration in human when applied as therapeutic agent. In contrast to clanobutin, the biguanides did not alter the rates of glucose production and lactate + pyruvate uptake significantly. However, addition of buformin caused a marked decrease in the rate of oxygen uptake whereas phenformin had no inhibitory effect on the rate of respiration.

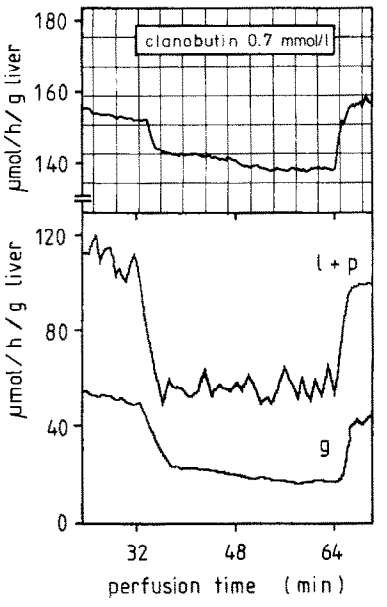

Fig. 1. Oxygen uptake, glucose output and lactate + pyruvate uptake in perfused liver from 22-hr fasted rat. Clanobutin was infused for 32 min (see block in upper figure). Upper panel—oxygen uptake. Lower panel—lactate + pyruvate uptake ($l + p$) and glucose output (g).

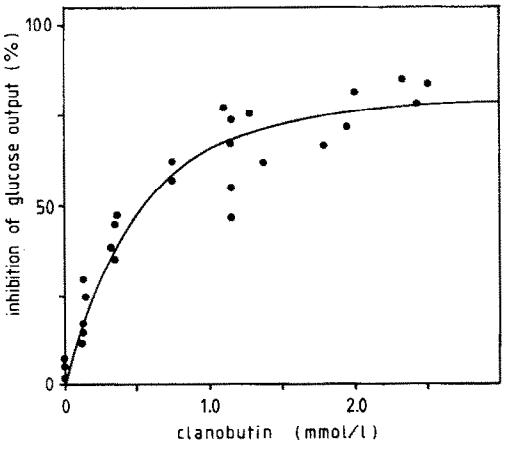

Fig. 2. Percentage inhibition of glucose output vs clanobutin concentration. Observed points (•); computer-fitted curve (—). Data from 27 perfusion experiments with livers from 22-hr fasted rats. Functional relationship: $y = y_{max}(1 - e^{-kx})$; $y_{max} = 81 \pm 3$ per cent; dose $(y_{max}/2) = 0.33 \pm 0.04$ mmoles/l, where dose $(y_{max}/2) = \ln 2/k$.

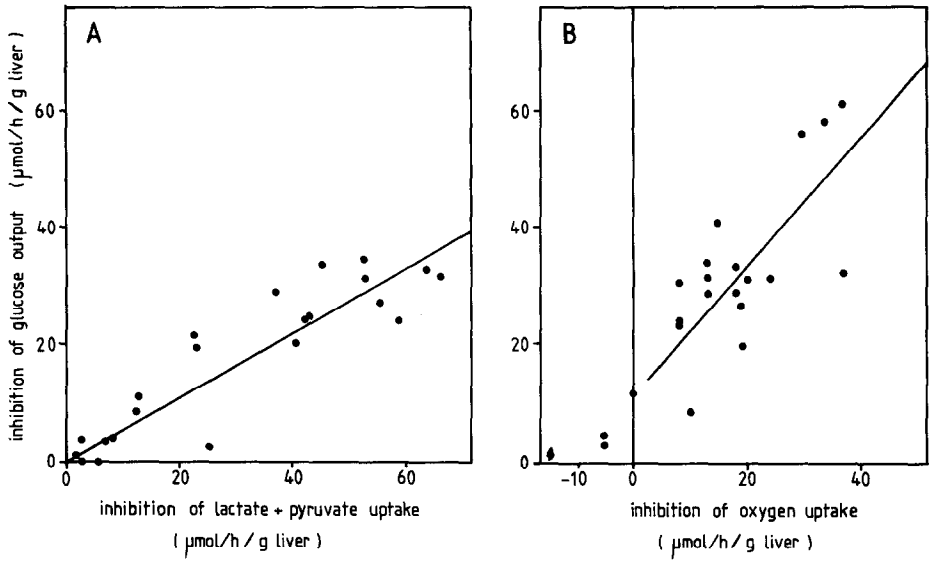

Fig. 3. Panel A: Inhibition of glucose output vs inhibition of lactate + pyruvate uptake. Data from perfusion experiments with livers from 22-hr fasted rats. The regression line follows the equation: $y = 0.55x$. Panel B: Inhibition of glucose output vs inhibition of oxygen uptake. Data from perfusion experiments with livers from 22-hr fasted rats. The regression line follows the equation: $y = 12 + 1.1x$.

On the other hand, clanobutin—applied at the 30-fold therapeutic plasma concentration in humans—caused a 66 per cent inhibition of gluconeogensis but a considerably smaller decrease in the respiratory rate than buformin.

DISCUSSION

In this study, clanobutin, an acylated anilino-alcanoic acid, was found to be capable of inhibiting gluconeogenesis. In isolated perfused livers of fasted rats, glucose synthesis from lactate + pyruvate was inhibited up to 81 per cent by clanobutin. The uptake of gluconeogenic precursors and oxygen was decreased in a stoichiometric manner (glucose : lactate + pyruvate : oxygen = 1 : 1.9 : 0.9 on a molar basis; see legend of Fig. 3). Thus, the data indicate that gluconeogenesis is not diminished due to mere diversion of the substrates into other pathways.

With regard to the mechanism of inhibitory effect, the following questions arise: Does clanobutin affect the enzymes in the gluconeogenic pathway directly or does it inhibit gluconeogenesis indirectly, for example, due to its own metabolism or due to changes in the redox state or in the rate of ATP-generating processes? At present, sufficient data are not available to answer these questions adequately. However, an indirect effect on gluconeogenesis either from redox changes or ATP-depletion as the result of an inhibition of the respiratory chain is unlikely for the following reasons: concentration ratios of lactate to pyruvate and β-hydroxybutyrate to acetoacetate in the perfusate are nearly unchanged (8 and 1.4, respectively) following addition of clanobutin. The rate of oxygen uptake was decreased by clanobutin; however, the decrease was stoichiometric to the diminished rate of glucose production. Moreover, the respiratory rate of isolated hepatic mitochondria in states 3 and 4, in the presence of succinate or malate and pyruvate, was not affected by clanobutin (J. Schlepper, unpublished observations).

Table 1. Inhibition of oxygen uptake, lactate + pyruvate uptake and glucose output by comparable doses of clanobutin, buformin and phenformin

	Dose	Inhibition (μmoles/hr/g)			Inhibition (%)
Substance	(mmoles/l)	Oxygen uptake	Lactate + pyruvate uptake	Glucose output	Glucose output
Clanobutin*	0.80	19 ± 4 (12; 27)	60 ± 7 (54; 66)	33 ± 4 (25; 41)	66 ± 4 (57; 75)
Buformin†	0.42 ± 0.07	45 ± 8 (21; 68)	13 ± 9 (−11; 36)	−2 ± 4 (−14; 9)	−3 ± 7 (−23; 16)
Phenformin†	0.13 ± 0.01	−8 ± 2 (−13; −3)	2 ± 2 (−5; 8)	−2 ± 3 (−10; 6)	−7 ± 5 (−21; 8)

* Predicted value ± S.E. derived from computer-fitted curve.
† $\bar{x}$ ± S.E.M. (N = 5).
The values given in parentheses refer to the 95 per cent confidence limits.

Measurements of the respiratory rate in the livers suggest that clanobutin is a substrate for mixed-function oxidation. For example, at low clanobutin concentrations only small changes in the rate of oxygen uptake were observed despite the fact that glucose production was inhibited (see Fig. 3b, intercept of the regression line), suggesting the superposition of two opposing mechanisms, i.e. increased oxygen consumption despite diminished gluconeogenesis. However, the rate of possible mixed-function oxidation of clanobutin appears to be too low to affect gluconeogenesis according to mechanisms previously suggested by Scholz *et al.* [11] of interactions between drug metabolism and biosynthetic processes.

In contrast to clanobutin, biguanides (buformin, phenformin) at comparable concentrations did not affect glucose production significantly (Table 1).

The diminished oxygen uptake under buformin is consistent with observations that biguanides are specific inhibitors of the respiration chain as shown with isolated mitochondria [23, 24]. According to Cook [25], phenformin in concentrations up to 1.24 mmoles/l had no significant effect on oxygen uptake in the perfused liver of fed rats. Thus, it is not surprising that the phenformin concentration of 0.13 mmoles/l which we used in our experiments did not produce an inhibition of oxygen uptake.

Moreover, the lack of substantial inhibition of gluconeogenesis by both biguanides indicates that, despite partial inhibition of the respiratory chain, ATP is sufficiently supplied to maintain gluconeogenesis. An explanation of the discrepancy in the magnitude of inhibitory effect of biguanides on gluconeogenesis, as observed by us and other authors, could be provided by the following facts:

First—most results on inhibition of gluconeogenesis by biguanides were obtained in guinea pigs. Rats, however, show a higher rate of biguanide metabolism than guinea pigs. They also show a different intracellular distribution of gluconeogenic key-enzymes in hepatocytes.

Second—the biguanide dose most frequently applied in rat liver perfusion experiments is 1 mmole/l or higher, thus reaching the dose range toxic in the intact rat.

Third—the considerably larger but delayed inhibitory effect of 1 mmole/l phenformin reported by Medina *et al.* [19] may be due to the accumulation of active metabolites in the recirculating perfusate. The question whether the effects of clanobutin are potentiated in a recirculating system is at present under investigation.

According to our knowledge, clanobutin appears to be more potent and probably more specific an inhibitor of gluconeogenesis than the biguanides, at least in the isolated perfused rat liver.

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