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INHIBITION OF HEPATIC GLUCONEOGENESIS BY METFORMIN

SYNERGISM WITH INSULIN

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Abstract—The antihyperglycaemic agent, metformin (dimethylbiguanide), inhibits hepatic gluconeogenesis. To investigate the mechanism involved, glucose production from collagenase-isolated hepatocytes of starved rats was determined after 1 hr incubations with different substrates. In the absence of insulin, glucose production from 10^{-2} M lactate–10^{-3} M pyruvate, 10^{-2} M alanine, 10^{-2} M glutamine and 5×10^{-3} M glycerol was decreased (35–78%) by high concentrations (10^{-2} and 10^{-3} M) of metformin. Lower concentrations of metformin were not effective in the absence of insulin, but a therapeutic concentration (10^{-5} M) of metformin acted synergistically with insulin (10^{-8} M) to suppress gluconeogenesis from each of the substrates by an additional 10–14% compared with insulin (10^{-8} M) alone. The synergistic antigluconeogenic effect of metformin (10^{-5} M) with insulin (10^{-8} M) was achieved without alteration of the contents of NADH and NAD^+ in digitonin-separated cytosolic and mitochondrial-rich hepatocyte fractions. Mitochondrial ATP was also unaltered by the metformin (10^{-5} M)–insulin (10^{-8} M) combination. However, the antigluconeogenic effect of 10^{-2} M metformin alone was associated with an increased (by 109%) mitochondrial NADH:NAD^+ ratio. Thus reduced gluconeogenesis by high concentrations of metformin (e.g. 10^{-2} M) may involve changes of redox state. However, therapeutic concentrations of metformin (e.g. 10^{-5} M) potentiate the antigluconeogenic effect of insulin to a similar extent from a range of substrates, without altering energy status or redox state.

Metformin (dimethylbiguanide) is an antihyperglycaemic agent used in the treatment of non-insulin dependent diabetes mellitus (NIDDM) [1]. It is reported to act by reducing the rate of intestinal glucose absorption, increasing peripheral glucose uptake and suppressing hepatic gluconeogenesis [2]. Studies in muscle and fat suggest that metformin improves insulin action [3–5], possibly due in part to increased insulin–receptor binding, but more importantly through an effect at the postreceptor level of insulin action [2, 6].

Heightened awareness that inappropriately raised hepatic glucose production makes an important contribution to the hyperglycaemia of NIDDM has promoted interest in antigluconeogenic agents [7]. Previous studies [8, 9] showing a direct inhibitory effect of metformin on hepatic gluconeogenesis were conducted in the absence of insulin, and used metformin concentrations considerably above the normal therapeutic range (which is plasma concentrations up to 5×10^{-5} M). Recently we noted that therapeutic concentrations of metformin can increase insulin–receptor binding in hepatocytes [10] and potentiate the suppression of gluconeogenesis by insulin [11]. Metformin has also been shown to restore insulin's ability to inhibit adenylate cyclase activity in liver plasma membranes of streptozotocin diabetic rats, although metformin did not alter insulin's ability to inhibit adenylate cyclase in liver plasma membranes of normal rats [12].

The present study further investigates the interaction between insulin and metformin in the control of gluconeogenesis by isolated hepatocytes. The study examines the effects of different gluconeogenic substrates, and the energy status and redox state of the cells.

MATERIALS AND METHODS

Animals and chemicals. Male Wistar rats weighing 200–300 g were used after 48 hr starvation. Collagenase (batch 16F-6805), crystalline bovine insulin (batch 55F-0536, 23.4 IU/mg) and enzymes and substrates for NAD^+ and NADH determinations were from Sigma Chemical Co. (Poole, U.K.). GOD-perid and ATP assay kits were from Boehringer Corporation Ltd (Lewes, U.K.), and metformin hydrochloride (batch 2452) was from Lipha Pharmaceuticals Ltd (West Drayton, U.K.). Other chemicals were of analytical grade and were obtained from Flurochem (Glossop, U.K.) and British Drug Houses (Poole, U.K.).

Isolated hepatocytes. Hepatocytes were isolated by the collagenase perfusion method [13, 14] using modifications described previously [11]. Cell viability, assessed by trypan blue exclusion, was $\geq 85\%$, and cell number was determined. Cells were preincubated for 15 min in Krebs–Ringer bicarbonate (KRB), gassed with 95% O_2 and 5% CO_2

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at 37° with continuous gentle agitation. Aliquots of the preincubated cell suspension were transferred to Erlenmeyer flasks (25 ml capacity) to give approximately 10^5 viable cells in a final volume of 5 ml. The gluconeogenic substrates (lactate–pyruvate, alanine, glutamine and glycerol), insulin and metformin were added at the beginning of the 1 hr incubation period at the concentrations documented in the Results section. Cells were incubated at 37°, continuously shaken and gassed at intervals.

Analyses. For glucose determination, a 100 μl aliquot of final incubation medium was transferred to 400 μl of 0.16% uranyl acetate, and centrifuged at 10,000 *g* for 45 sec. The supernatant was assayed by the glucose oxidase method [15]. Glucose production was calculated as nmoles/10^5 viable cells/min, and expressed as a percentage of the control value (100%) determined in the same series of experiments.

Alanine uptake was measured by incubation of hepatocytes as above in KRB containing 10^{-2} M alanine. Cells were separated by centrifugation, and 0.5 ml of cell-free supernatant was added to 0.5 ml 12% perchloric acid, mixed, recentrifuged and neutralised with 2 M KOH. Alanine was assayed by the alanine dehydrogenase method [16].

NADH and NAD^+ were measured in supernatant (cytosolic) and pellet (mitochondrial-rich) fractions, and ATP was measured in the pellet fractions which were rapidly prepared using a digitonin separation method [17, 18]. A 200 μl aliquot of cells was mixed for 15 sec with 1.2 ml ice-cold digitonin buffer, pH 7.0, comprising 250 mM sucrose, 20 mM potassium MOPS, 3 mM EDTA and 4 g/l digitonin. The suspension was centrifuged at 10,000 *g* for 10 sec, and 1 ml of supernatant was transferred to 0.2 ml of 72% (w/w) perchloric acid, giving a final concentration of 12% perchloric acid. The pellet was acidified to a final concentration of 12% perchloric acid. Acidified fractions were neutralised with 2 M KOH, recentrifuged at 10,000 *g* for 45 sec and the neutralised extracts stored at −20°. ATP was measured by the phosphoglycerate kinase method [19], NAD^+ by the alcohol dehydrogenase method and NADH by the glycerol-3-phosphate dehydrogenase method [20]. The presence of metformin did not interfere with these assay methods. It may be worthy of note here that metformin can interfere with ATP measurements using a luciferase method (Wollen and Bailey, unpublished observations).

Statistics. Groups of data were compared using Student's unpaired *t*-test. Differences were considered to be significant if $P < 0.05$. If the variances of the means of the control and experimental groups were significantly different ($P < 0.05$ using the variance ratio F-test), the significance of difference between the means of these groups was reassessed using the Fisher–Behrens test.

RESULTS

Gluconeogenesis without insulin

Glucose production during 1 hr incubations of isolated hepatocytes without added insulin varied with the gluconeogenic substrate supplied. For example, control values for glucose production (nmoles/10^5 viable cells/min, mean ± SEM, N = 18) from each of the substrates illustrated in Fig. 1 were: 42.9 ± 2.2 from 5×10^{-3} M glycerol; 32.5 ± 2.5 from 10^{-2} M lactate–10^{-3} M pyruvate; 8.4 ± 0.5 from 10^{-2} M glutamine, and 8.0 ± 0.6 from 10^{-2} M alanine. Metformin concentrations of 10^{-2} and 10^{-3} M reduced glucose production by 78–35% ($P < 0.001$ compared with control for the same substrate) for each of the substrates tested (Fig. 1). Lower concentrations of metformin (10^{-4} and 10^{-5} M) did not exert a significant effect with any of the substrates.

To investigate whether the lack of effect of the lower metformin concentrations might be due to insufficient time, hepatocytes were preincubated for 1 hr with 10^{-4} M metformin prior to test incubation for 1 hr with the same concentration of metformin (Fig. 2). The additional period of exposure to met-

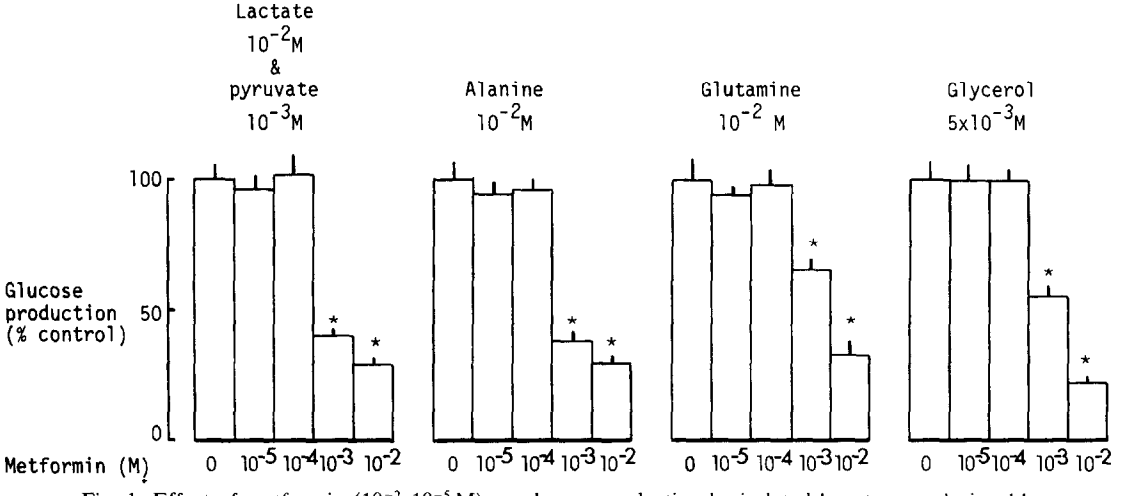

Fig. 1. Effect of metformin (10^{-2}–10^{-5} M) on glucose production by isolated hepatocytes during 1 hr incubations in the absence of added insulin. Values are mean ± SEM; N = 18 for controls, N = 6 for all other values. * $P < 0.001$ compared with control (no metformin).

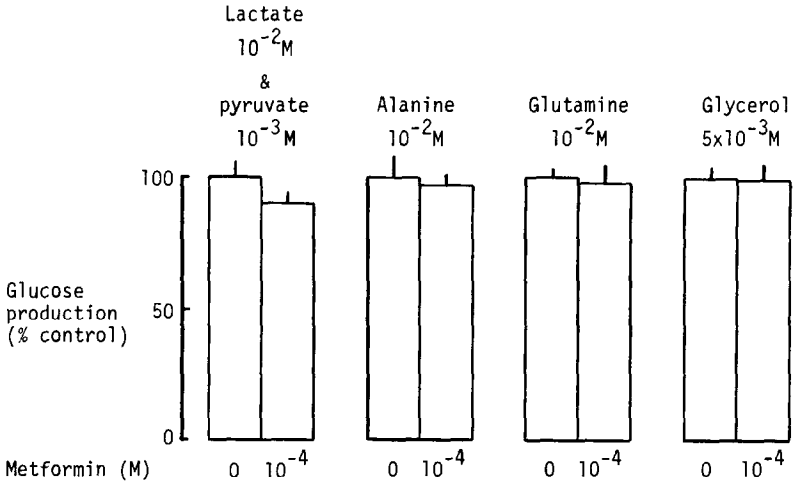

Fig. 2. Effect of 1 hr preincubation and 1 hr incubation with metformin (10^{-4} M) on glucose production by isolated hepatocytes in the absence of added insulin. Values are mean ± SEM, N = 6.

formin did not significantly affect glucose production from any of the substrates tested.

Gluconeogenesis with insulin

Incubation of hepatocytes for 1 hr with addition of 10^{-8} M insulin reduced glucose production from lactate–pyruvate (28%) to a greater extent than from glutamine (16%) and alanine (12%) (lactate–pyruvate compared with glutamine and alanine, $P < 0.05$) (Fig. 3). At 10^{-8} M, insulin did not significantly alter glucose production from glycerol. 10^{-5} M metformin alone did not affect glucose production from any of the substrates tested, but this concentration of metformin in combination with 10^{-8} M insulin reduced glucose production to a greater extent than insulin alone. The further suppression of glucose production by metformin in the presence of insulin was similar for each of the substrates tested (lactate–pyruvate 14%; glutamine 13%; alanine 11%; glycerol 10%).

Alanine uptake

Since the rate of gluconeogenesis from alanine may be regulated in part by adjustments of alanine transport into hepatocytes [21], the effect of metformin and insulin on hepatocyte alanine accumulation was examined (Table 1). During 1 hr incubations with 10^{-2} M alanine, the presence of metformin (10^{-2} and 10^{-5} M), insulin (10^{-8} M) or a combination of insulin (10^{-8} M) with metformin (10^{-5} M) did not affect hepatocyte alanine accumulation.

NADH, NAD⁺ and ATP

NADH and NAD^+ contents of digitonin-separated supernatant and pellet fractions of hepa-

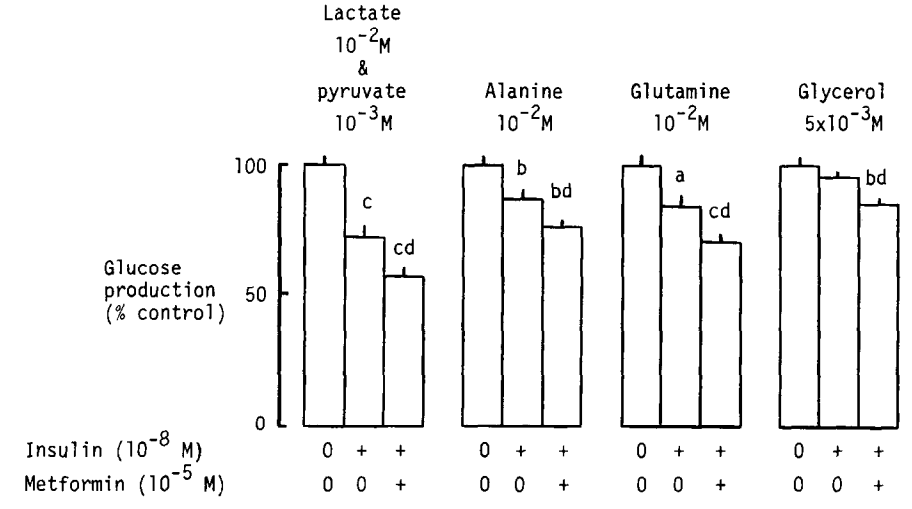

Fig. 3. Effect of insulin (10^{-8} M) and metformin (10^{-5} M) on glucose production by isolated hepatocytes during 1 hr incubations. Values are mean ± SEM, N = 6. $^aP < 0.05$, $^bP < 0.01$, $^cP < 0.001$ compared with control (no insulin and no metformin). $^dP < 0.05$ compared with insulin alone.

Table 1. Effect of metformin and insulin on alanine accumulation by isolated hepatocytes of starved rats during 1 hr incubations with 10^{-2} M alanine

	Alanine accumulation nmoles/10^5 viable cells/min
a.	
Control	44.1 ± 4.0
Metformin 10^{-2} M	46.9 ± 10.5
Metformin 10^{-5} M	54.8 ± 6.2
b.	
Control	52.7 ± 6.6
Insulin 10^{-8} M	47.6 ± 6.9
Insulin 10^{-8} M and Metformin 10^{-5} M	53.1 ± 9.5

Values are mean ± SEM, N = 6.

tocytes were examined after 1 hr incubation with lactate–pyruvate as substrate (Table 2). The NADH and NAD^+ contents of both fractions were not significantly altered by the incubations with metformin (10^{-5} M) and insulin (10^{-8} M) alone and in combination. However 10^{-2} M metformin increased (by 71%) the NADH content and increased (by 109%) the NADH:NAD^+ ratio of the pellet fraction. The ATP content of the pellet fraction was not significantly altered by metformin (10^{-2} and 10^{-5} M), insulin (10^{-8} M), or a combination of insulin (10^{-8} M) with metformin (10^{-5} M) (Table 3). In a parallel series of experiments it was confirmed that glucose production was reduced (67%) by 10^{-2} M metformin, but unaffected by 10^{-5} M metformin, although the latter concentration of metformin again accentuated by 14% the reduction of glucose production by 10^{-8} M insulin.

DISCUSSION

Metformin is the only biguanide antihyperglycaemic agent used in most countries. The other main biguanide phenformin (phenethylbiguanide) was discontinued due to an association with lactic acidosis [22]. High concentrations ($> 10^{-3}$ M) of phenformin inhibit hepatic gluconeogenesis in the absence of insulin [8, 9, 23, 24] but the effects of therapeutic concentrations of phenformin and

Table 2. Effect of metformin and insulin on NADH and NAD^+ contents of the supernatant and pellet fractions of isolated rat hepatocytes*

	Supernatant			Pellet		
	NADH	NAD^+	NADH:NAD^+	NADH	NAD^+	NADH:NAD^+
Control	74 ± 8	49 ± 7	1.5 ± 0.2	28 ± 3	24 ± 2	1.1 ± 0.1
Metformin 10^{-2} M	59 ± 13	35 ± 8	1.6 ± 0.7	48 ± 5[a]	20 ± 4	2.3 ± 0.2[b]
Metformin 10^{-5} M	77 ± 21	47 ± 9	1.6 ± 0.4	33 ± 3	27 ± 4	1.2 ± 0.3
Control	80 ± 19	55 ± 17	1.4 ± 0.3	37 ± 5	22 ± 2	1.6 ± 0.1
Insulin 10^{-8} M	80 ± 27	69 ± 10	1.1 ± 0.2	47 ± 11	27 ± 5	1.7 ± 0.2
Insulin 10^{-8} M and Metformin 10^{-5} M	80 ± 15	62 ± 8	1.3 ± 0.2	49 ± 7	25 ± 5	1.8 ± 0.3

* NADH and NAD^+ values expressed as nmol/10^5 viable cells.
Results are mean ± SEM of 6 determinations.
[a] $P < 0.05$, [b] $P < 0.01$ compared with control.

Table 3. Effect of metformin and insulin on ATP content of the pellet fractions of isolated rat hepatocytes

	ATP nmol/10^5 viable cells
Control	1.93 ± 0.05
Metformin 10^{-2} M	1.97 ± 0.08
Metformin 10^{-5} M	1.89 ± 0.12
Control	2.17 ± 0.34
Insulin 10^{-8} M	2.20 ± 0.30
Insulin 10^{-8} M and Metformin 10^{-5} M	1.86 ± 0.46

Results are mean ± SEM of 6 determinations.

the interaction of phenformin and insulin in the control of hepatic gluconeogenesis have not been examined. It has been suggested that the antigluconeogenic effect of a high concentration of phenformin may result in part from decreased oxidative phosphorylation and an increased NADH:NAD^+ ratio, but a direct relationship has not been observed [23–25].

Quantitative and qualitative differences in the pharmacokinetic properties and metabolic effects of metformin and phenformin necessitate careful discrimination between these two biguanides. With regard to hepatic metabolism, metformin is not altered chemically whereas phenformin is hydroxylated [26]. The affinity with which metformin binds to mitochondrial membranes is about 2% that of phenformin [25], and metformin does not exhibit the strong inhibitory effect on oxidative phosphorylation shown by phenformin [27].

The present study confirmed that in the absence of insulin only high concentrations (10^{-3} M and above) of metformin reduced hepatic gluconeogenesis. However, a therapeutic concentration (10^{-5} M) of metformin, which was ineffective in the absence of insulin, acted synergistically with insulin (10^{-8} M) to reduce hepatic glucose production. The similar extent to which metformin reduced hepatic gluconeogenesis from each of the substrates examined (lactate–pyruvate, glycerol, alanine and glutamine) suggests a similar mode of action. Changes in substrate availability are unlikely, because each of the substrates used is readily accessible to hepatocytes at the concentrations used [28]. Also, it was demonstrated that the accumulation of alanine by hepatocytes was not affected by the drug. Moreover, the mitochondrial ATP content of the cells, which offers a guide to energy status, did not appear to be a limiting factor. The rapidity of the antigluconeogenic effect of metformin suggests an alteration of enzyme activity, but the precise location of action cannot be deduced from the present study. It is envisaged that in the absence of insulin, high concentrations of metformin decrease gluconeogenesis by a different mechanism to therapeutic concentrations of metformin acting with insulin. For example the antigluconeogenic effect of a high concentration (10^{-2} M) of metformin was associated with an increased mitochondrial NADH:NAD^+ ratio, but this did not account for the synergistic antigluconeogenic effect of a therapeutic concentration of metformin with insulin. The similar extent to which metformin reduced gluconeogenesis from glycerol and the other substrates tested raises the possibility of an effect between glyceraldehyde-3-phosphate and glucose. However, a proportion of glycerol will enter glycolysis [29]. Thus the present data do not discount a regulatory action at earlier cytosolic or mitochondrial steps in the gluconeogenic pathway.

Potentiation of an insulin-sensitive regulatory step in gluconeogenesis, by an effect at either receptor or postreceptor sites of insulin action, provides a mechanism through which therapeutic concentrations of metformin can ameliorate hepatic insulin resistance and reduce glucose production in diabetes mellitus.

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