Evidence that metformin exerts its anti-diabetic effects through inhibition of complex 1 of the mitochondrial respiratory chain

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Although metformin is widely used for the treatment of non-insulin-dependent diabetes, its mode of action remains unclear. Here we provide evidence that its primary site of action is through a direct inhibition of complex 1 of the respiratory chain. Metformin (50 μM) inhibited mitochondrial oxidation of glutamate + malate in hepatoma cells by 13 and 30 % after 24 and 60 h exposure respectively, but succinate oxidation was unaffected. Metformin also caused time-dependent inhibition of complex 1 in isolated mitochondria, whereas in sub-mitochondrial particles inhibition was immediate but required very high metformin concentrations ($K_{0.5}$, 79 mM). These data are compatible with the slow membrane-potential-driven accumulation of the positively charged drug within the mitochondrial matrix leading to inhibition of complex 1. Metformin inhibition of gluconeogenesis from L-lactate in isolated rat hepatocytes was also time- and concentration-dependent, and accompanied by changes in metabolite levels similar to those induced by other inhibitors of gluconeogenesis acting on complex 1. Freeze-clamped livers from metformin-treated rats exhibited similar changes in metabolite concentrations. We conclude that the drug's pharmacological effects are mediated, at least in part, through a time-dependent, self-limiting inhibition of the respiratory chain that restrains hepatic gluconeogenesis while increasing glucose utilization in peripheral tissues. Lactic acidosis, an occasional side effect, can also be explained in this way.

Key words: gluconeogenesis, glucose utilization, lactic acidosis, mitochondria, NIDDM.

INTRODUCTION

The biguanide, metformin (N^1,N^1-dimethylbiguanide), is used extensively to treat non-insulin-dependent diabetes mellitus (NIDDM), yet its primary locus of action remains uncertain. Major effects of the drug in humans are to decrease hepatic glucose output, especially gluconeogenesis from L-lactate, and to increase both glycolytic lactate production by the intestine and insulin-dependent peripheral glucose utilization [1–3]. The latter process is associated with increased plasma-membrane expression of glucose-transporter isoforms GLUT1 and GLUT4 [4–7].

Early studies showed that some alkylguanidines, which are related to the biguanides, can inhibit the oxidation of glutamate + malate but not succinate by isolated energized liver mitochondria, although their exact locus and mode of action was not elucidated [8,9]. Although direct effects of phenformin and metformin on the respiratory chain of isolated mitochondria have not been reported, mitochondria isolated from the livers of guinea pigs treated with phenformin were shown to exhibit decreased rates of 2-oxoglutarate oxidation but not succinate oxidation [10]. Thus it was suggested by some workers that biguanides might inhibit hepatic gluconeogenesis through inhibition of mitochondrial respiration and ATP production [8,9,11,12]. However, direct measurements of tissue ATP content did not support this view [13] and most workers now regard the effects of metformin on mitochondria as being responsible only for side effects of the drug, such as occasional lactic acidosis.

We decided to re-examine the possibility that inhibition of mitochondrial respiration might be a primary site of action of metformin, since previous work from this laboratory has demonstrated that the respiratory chain has a high flux-control coefficient for gluconeogenesis. Indeed, we showed that a modest inhibition of respiration by the mild respiratory-chain inhibitors 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) or amytal caused a substantial inhibition of gluconeogenesis without significant changes in total tissue ATP content [14,15]. Furthermore, activation of the respiratory chain by glucagon is thought to play an important role in the stimulation of gluconeogenesis by this hormone [14]. Thus a drug that produces mild inhibition of the respiratory chain could be an effective hypoglycaemic agent. In this paper we provide strong evidence that this is the case for metformin and phenformin that directly inhibit complex 1 of the respiratory chain in a time-dependent, self-limiting manner. We propose that this inhibition restrains hepatic gluconeogenesis while increasing glucose utilization in peripheral tissues and may also explain why lactic acidosis is an occasional side effect of treatment [3]. After completion of this work, a study by El-Mir et al. [16] also demonstrated inhibition of complex 1 in hepatocytes and perfused livers by high concentrations (1–10 mM) of metformin, but proposed that this effect was exerted indirectly by some unidentified signalling pathway.

EXPERIMENTAL

Animals, chemicals, drugs and reagents

Male Wistar rats (250–300 g in weight) were used for preparation of both hepatocytes and liver mitochondria, and for studies *in vivo*.

Metformin, phenformin and antibiotics were obtained from Sigma (Poole, Dorset, U.K.). Cell-culture media were from Gibco-BRL (Paisley, U.K.). The sources of all other chemicals and biochemicals were as given previously [14,15].

Preparation and incubation of isolated mitochondria and sub-mitochondrial particles (SMPs)

Isolated rat liver mitochondria were prepared from 24-h-starved rats and further purified by Percoll gradient centrifugation to remove microsomal and plasma-membrane contamination as

Abbreviations used: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; SMP, sub-mitochondrial particle.

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described previously [15]. Heart mitochondria were prepared from fed rats using a similar procedure but incorporating protease treatment before homogenization [17]. SMPs were prepared by sonication of either heart or liver mitochondria as described elsewhere [18]. Respiration by isolated mitochondria (1 mg of protein/ml) was studied at 30 °C using an oxygen electrode [17,19]. The medium contained 125 mM KCl, 20 mM Mops, 10 mM Tris, 0.5 mM EGTA, 2 mM potassium phosphate and 2 mM $MgCl_2$ (pH 7.2). The substrate was either 5 mM L-glutamate + 1 mM L-malate or 5 mM succinate + 1 μM rotenone, and respiration was stimulated by additions of 0.4 mM ADP (state 3). Oxidation of NADH by SMPs (75 μg of protein/ml) was measured at 30 °C in the same buffer containing 0.15 mM NADH; the decrease in [NADH] was measured continuously at 340 nm in a split-beam spectrophotometer.

Culture, permeabilization and measurement of mitochondrial respiration in rat hepatoma cells

Rat hepatoma cells (H4IIE) were grown under air/CO_2 (19:1) in Ham's F-12 medium, supplemented with 5% foetal bovine serum, 2 mM L-glutamine and 2 mM penicillin/streptomycin and were maintained in logarithmic phase throughout. Metformin (final concentration 50 or 100 μM from a 25 mM stock solution in water) was added to cells for 24 or 60 h prior to harvesting, with water alone added to controls. Once harvested, cells were washed twice with media containing 150 mM NaCl, 5 mM KCl, 10 mM glucose and 10 mM Tris, pH 7.4, and then suspended in ice-cold medium containing 210 mM mannitol, 70 mM sucrose, 10 mM glucose, 5 mM Hepes and 5 mg/ml defatted BSA, pH 7.2. Cells were then permeabilized according to Bogucka et al. [20]. Digitonin (0.2 mg/ml, final concentration) was added for 1–2 min at 0 °C until > 90% of the cells became permeable to Trypan Blue. The reaction was stopped by adding 2 vol. of the mannitol/sucrose medium and the cells sedimented at 3000 ***g*** for 3 min before resuspending in the mannitol/sucrose medium at 8–12 mg of protein/ml. Respiration was measured in a Clark-type oxygen electrode as described above.

Preparation and incubation of isolated hepatocytes

Hepatocytes were isolated from 24-h-starved rats by collagenase digestion as described previously [14,15] and incubated at 5 mg of protein/ml in bicarbonate-buffered saline medium containing 10 mM L-lactate, 1 mM pyruvate, 0.3 μM glucagon and 20 mg/ml defatted BSA, with a gas phase of O_2/CO_2 (19:1). Glucagon was added to provide conditions of stimulated gluconeogenesis and fatty acid oxidation such as occur in diabetes. Measurements of the rates of gluconeogenesis and metabolite concentrations were performed as described previously [15].

Metformin treatment of rats and measurement of metabolites in freeze-clamped livers

Oral intubation was used to deliver doses (1 ml) of either metformin (50 or 150 mg/ml in water) or water alone directly into the stomachs of male Wistar rats (250–300 g in weight). Animals were treated once each day for 5 days. They were allowed free access to water at all times, but food was removed 24 h before the final treatment. The treatments were carried out according to Home Office regulations and did not cause the animals any visible distress or significantly affect mean body weight. It should be noted that the doses of metformin used in the present study are typical of those shown by many workers to be required for the demonstration of therapeutic effects in rats and mice [21–25]. Following the final treatment (4 h), animals were anaesthetized and their livers rapidly removed and divided to provide material for both the preparation of mitochondria (as described above) and for freeze-clamping. Freeze-clamped material was ground in a pestle and mortar pre-cooled with liquid nitrogen. The ground material (1 g) was mixed with ice-cold perchloric acid [4 ml of 5% (w/v)] and homogenized using a Polytron blender (15 s at setting 3). Precipitated protein was removed by centrifugation and aliquots of the acid supernatant neutralized and assayed for various metabolites as described previously [15].

Statistics

The significance of differences between control and experimental groups were performed using a two-tailed Student's t test.

RESULTS

Inhibition of mitochondrial respiration by metformin in permeabilized rat hepatoma cells

Rat hepatoma cells were cultured with 50 and 100 μM metformin for either 24 or 60 h and then, following digitonin permeabilization, the rates of their ADP-stimulated mitochondrial respiration were measured. Data are shown in Figure 1. No effect of metformin on succinate oxidation was observed, whereas rates of glutamate + malate oxidation were reduced significantly. Metformin (50 μM) caused 12.6 ± 4.3 and 29.4 ± 7.7% inhibition after 24 and 60 h, respectively, whereas for 100 μM metformin the inhibition was 25.8 ± 3.4 and 37.1 ± 10.0%, respectively. Control ratios (the ratio of respiration rates in the presence and absence of ADP) were 3.5 ± 0.24 for glutamate + -

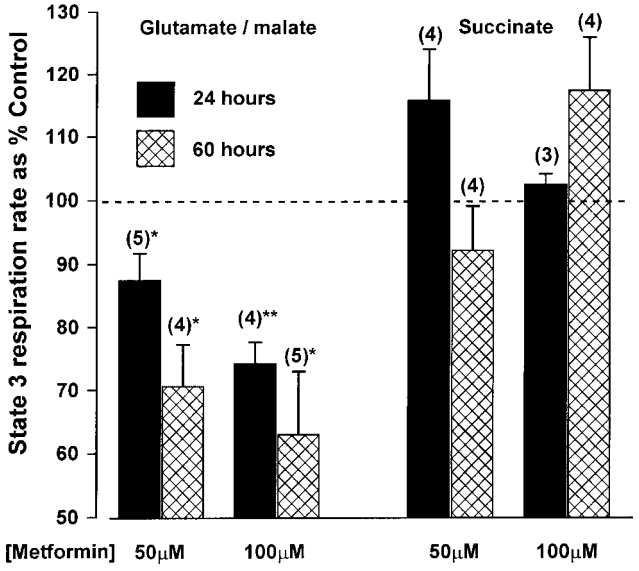


Figure 1 Inhibition by metformin of mitochondrial respiration in permeabilized rat hepatoma cells

Rat hepatoma (H4IIE) cells were cultured for 24 or 60 h in the presence or absence of metformin at 50 or 100 μM, before digitonin permeabilization and measurement of respiration as described in the Experimental section. Rates of ADP-stimulated respiration in the presence of metformin are expressed as a percentage of control rates (no metformin) and as means ± S.E.M. of the number of observations on separate cell preparations indicated in parentheses. Absolute rates of ADP-stimulated respiration in the absence of metformin were 16.8 ± 0.3 nmol of O atoms/min per mg of cell protein for glutamate + malate ($n = 18$) and for succinate oxidation 15.8 ± 0.43 ($n = 15$). The statistical significance of the effect of metformin treatment was determined by a paired Student's t test (*$P < 0.05$; **$P < 0.01$).

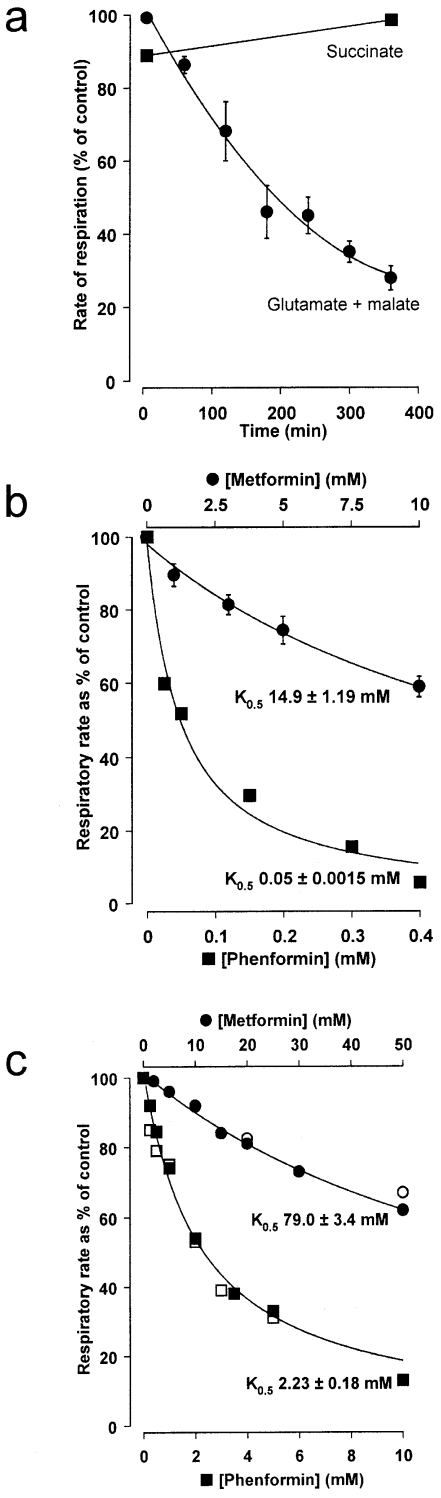


Figure 2 Metformin and phenformin inhibit NADH-dependent respiration by isolated mitochondria and SMPs

(**a**) Rat liver mitochondria (40 mg/ml) were incubated with gentle orbital shaking at 8 °C in isolation medium containing 10 mM metformin for the times shown. The rate of ADP-stimulated respiration in the presence of glutamate + malate or succinate + rotenone was then determined. (**b**) The inhibition following pre-incubation for 255 min with metformin at the concentrations shown is compared with that produced by phenformin following pre-incubation for 5 min. Data are expressed as a percentage of the control rate (incubated for the same time without drug). (**c**) The concentration dependence of the inhibition by phenformin and metformin of NADH oxidation by SMPs is shown. In (**a**) and (**b**) data for metformin are given as means ± S.E.M. of three separate mitochondrial preparations whereas for phenformin a single experiment is shown. In (**c**) the open and closed symbols represent preparations of SMPs from heart and liver mitochondria respectively. The $K_{0.5}$ values were derived by non-linear least-squares regression analysis using the equation $v\% = 100/(1 + I/K_{0.5})$, where I is the concentration of inhibitor and v% is the rate of respiration in the presence of the drug as a percentage of the rate in its absence.

malate and 2.5 ± 0.16 for succinate and were not significantly changed by metformin treatment. These data are consistent with a time-dependent inhibition of complex 1 and this was investigated further using isolated rat liver mitochondria.

Metformin and phenformin inhibit complex 1 in isolated mitochondria and SMPs

Preliminary experiments showed that addition of metformin to rat liver mitochondria oxidizing 5 mM L-glutamate + 1 mM L-malate in the presence of ADP gave no inhibition over a period of 5 min at 30 °C, even at concentrations as high as 10 mM. However, the data from the hepatoma cells suggested that the inhibition by metformin may take a long time to develop, perhaps indicating a very slow permeation of the drug into the mitochondria. We found that incubation of isolated mitochondria at physiological temperatures for periods longer than 10 min is not possible without considerable loss of mitochondrial integrity. Thus we decided to incubate at low temperature even though this might further reduce the rate of metformin permeation. We show in Figure 2(a) that incubation of mitochondria at 8 °C with 10 mM metformin produced progressive inhibition of the ADP-stimulated oxidation of glutamate + malate, but not of succinate, and even after 6 h inhibition was still increasing. The concentration dependence of this inhibition (after 255 min of pre-incubation at 8 °C) is shown in Figure 2(b), but it is important to note that much greater inhibition would be predicted if incubation with the inhibitor could have been continued for longer. Thus the true $K_{0.5}$ value for metformin will be substantially lower than indicated by the data shown. In contrast, the phenyl and ethyl groups on phenformin (N^1,N^1-phenylethylbiguanide) make it a more lipophilic drug that can permeate biological membranes readily, and this is reflected in the ability of this drug to exert its maximum inhibitory effect on glutamate + malate oxidation within 5 min at 30 °C. The concentration dependence of this inhibition is shown in Figure 2(b) and yields a $K_{0.5}$ value of 50 μM. Again, no effect of the drug on succinate oxidation was observed, implying that inhibition of respiration was occurring, either at the level of the dehydrogenases producing NADH, as has been suggested by others [9,10], or at complex 1 of the respiratory chain.

In order to discriminate between these possibilities, SMPs were used. These particles have the matrix surface of the respiratory chain accessible to the medium and unlike mitochondria they can oxidize added NADH directly. Furthermore, there is no permeability barrier to restrict access of metformin and phenformin to the matrix surface of the respiratory chain. Indeed, we found that NADH oxidation by SMPs was inhibited immediately upon addition of either drug with $K_{0.5}$ values of 79 and 2.2 mM, respectively (Figure 2c). Exposure of the SMPs to the drug for longer time periods did not increase the inhibition observed (results not shown). We have confirmed that inhibition of complex 1 is not confined to liver mitochondria by demonstrating that there was no difference in the sensitivity to metformin inhibition of respiration by SMPs from liver (Figure 2c, closed symbols) and heart (Figure 2c, open symbols). It is clear that the concentrations of the two drugs required to inhibit respiration in SMPs are substantially higher than for mito-

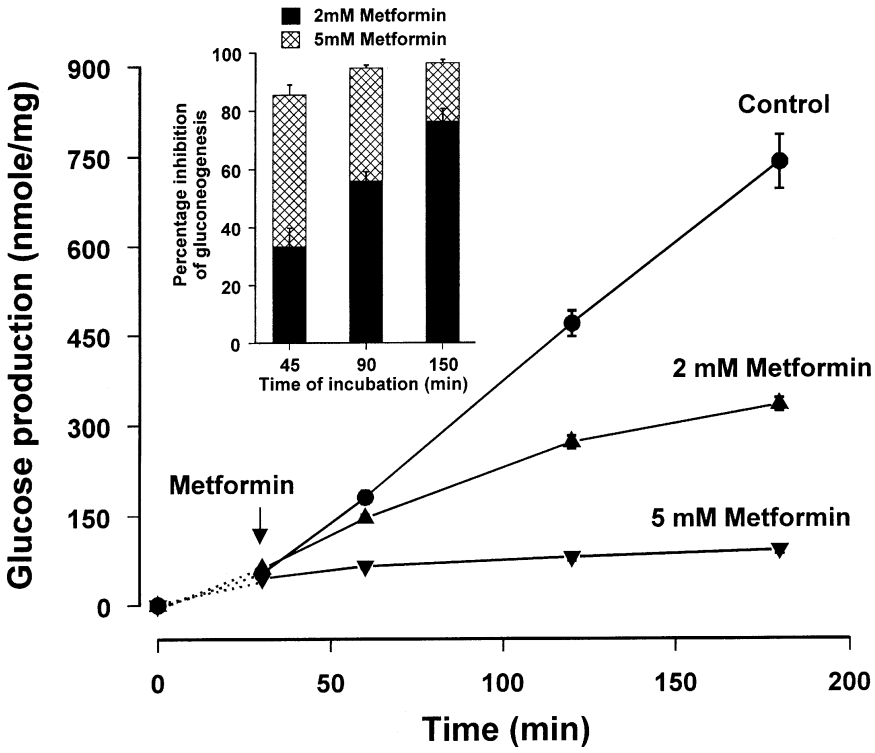


Figure 3 Inhibition of gluconeogenesis by metformin in isolated rat hepatocytes

Glucose production was determined after incubation of hepatocytes from 24-h-starved rats for the time shown in the presence of 10 mM L-lactate, 1 mM pyruvate, 20 mg/ml defatted BSA and 0.3 μM glucagon. Metformin at the concentrations shown was added as indicated by the arrow. The inset shows the progressive inhibition of gluconeogenesis caused by incubation with two concentrations of metformin for the times indicated.

chondria. This is most readily explained by a membrane-potential-driven accumulation of the positively charged drug within the mitochondria (see the Discussion section).

Changes in hepatocyte metabolite concentrations associated with the inhibition of gluconeogenesis by metformin

The data of Figure 3 show that metformin inhibits gluconeogenesis from L-lactate in isolated hepatocytes in a time- and concentration-dependent fashion. This is consistent with the time-dependent accumulation of the drug within the mitochondria and subsequent inhibition of the respiratory chain described above. After 2.5 h of incubation with 5 mM metformin inhibition of gluconeogenesis was almost total, whereas inhibition by 2 mM metformin had reached only 75%, but was still increasing. Although it is probable that inhibition of gluconeogenesis could have been obtained at substantially lower concentrations of metformin if hepatocytes had been incubated with the drug for longer, this was not a viable option. Control rates of gluconeogenesis were found to decline substantially when incubations were continued for long periods, implying that the integrity of the hepatocytes was not maintained.

In Figure 4 we report changes in the total hepatocyte content of some key metabolites of the gluconeogenic pathway following incubation with 2 mM metformin for 3 h. The concentrations of 2- and 3-phosphoglycerate and phosphoenolpyruvate increased by 411 and 336% respectively, whereas the concentrations of glucose 6-phosphate and fructose 6-phosphate decreased by about 50%. In addition, increases in the ratios of β-hydroxybutyrate/acetoacetate (349%) and L-lactate/pyruvate (266%) were found. These changes were all very similar to those we observed previously in isolated hepatocytes treated with the mild respiratory-chain inhibitors DCMU and amytal [14,15,26] and by others using phenformin [11]. Although previous work has demonstrated inhibition of gluconeogenesis by metformin in isolated rat hepatocytes [27,28] and perfused livers [29,30], detailed determination of the changes in metabolite concentrations were not made.

Effects of oral treatment of rats with metformin on hepatic metabolite concentrations

In order to demonstrate that metformin may act to inhibit gluconeogenesis *in vivo* through the same mechanism as observed in isolated hepatocytes, 250–300-g rats were treated by oral intubation for a period of 5 days with either 50 or 150 mg of metformin given once daily. It is recognized that even the lower dose (150–200 mg/kg) is some 4–5 times greater than the maximal daily dose of about 3 g used in humans [2,3]. However, it is well established that these relatively high doses of metformin are necessary for the drug to reach plasma concentrations in rats similar to those found in humans and to exert its hypoglycaemic effects in diabetic rats [21–25]. Wilcock and Bailey [25] have shown that in mice treated with 50 mg/kg metformin, portal and inferior vena cava concentrations of the drug peaked at 52 and 29 μM, respectively, there being no significant differences between control and diabetic mice. However, these values declined to $< 2\%$ after 24 h. Thus the concentrations used in our studies probably are similar to those found therapeutically in humans. The species difference in drug dosage required may

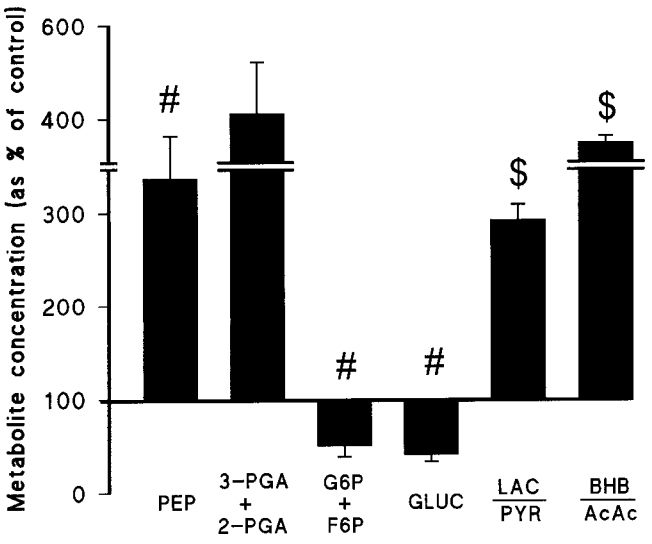


Figure 4 Changes in key metabolite concentrations and ratios after incubation of hepatocytes with 2 mM metformin for 3 h

Following incubation, hepatocytes were separated from the medium by centrifugation through oil into perchloric acid and metabolites measured in the neutralized extracts and supernantants [9]. Data in the presence of metformin are expressed as a percentage of the control value in the absence of metformin and as means ± S.E.M. of values for three separate cell preparations. Control values were similar to those reported previously [9]. The statistical significance of the effects of metformin were determined by a paired Student's *t* test (#$P < 0.05$; \$$P < 0.01$). PEP, phosphoenolpyruvate; 2- and 3-PGA, 2- and 3-phosphoglycerate; G6P, glucose 6-phosphate; F6P, fructose 6-phosphate; GLUC, glucose; LAC/PYR, L-lactate/pyruvate; BHB/AcAc, β-hydroxybutyrate/acetoacetate.

reflect a difference in the intestinal absorption and renal clearance of the drug [21–25].

After the final drug administration (4 h), livers were removed, freeze-clamped and extracted with perchloric acid for analysis of metabolites. The changes in glycolytic metabolite concentrations are shown in Figure 5 and are very similar to those found in isolated liver cells (Figure 4). Thus increased concentrations of 2- and 3-phosphoglycerate (257 and 332 % at 50 and 150 mg of metformin respectively) and phosphoenolpyruvate (309 and 535 %) and decreased concentrations of glucose 6-phosphate (60 and 61 %) and fructose 6-phosphate (63 and 33 %) were observed, whereas the ratios of both β-hydroxybutyrate/acetoacetate (233 and 403 %) and L-lactate/pyruvate (153 and 204 %) increased. Total cell concentrations of ATP, ADP and AMP showed no changes at either concentration of metformin. Control values for ATP, ADP and AMP (expressed as nmol/mg of protein, mean ± S.E.M., for four separate rat livers) were 8.81 ± 0.21, 4.97 ± 0.30 and 2.27 ± 0.19 respectively, whereas the values after metformin treatment at 50 mg daily were 8.57 ± 0.76, 5.05 ± 0.26 and 2.68 ± 0.17 respectively, and after metformin treatment at 150 mg daily were 9.00 ± 0.21, 5.09 ± 0.13 and 2.44 ± 0.01 respectively.

These values represent total tissue concentrations of the adenine nucleotides whereas metabolic control is exerted through the free metabolite concentrations. Thus in Figure 5 we also present data for the ratio for the free concentrations of ATP and ADP calculated from metabolite concentrations and the equilibrium constants of the reactions catalysed by lactate dehydrogenase, phosphoglyceraldehyde dehydrogenase and phosphoglycerate kinase [15]. The assumptions behind the use of these calculations have been verified in rat liver [31] and we have confirmed that 10 mM metformin does not inhibit any of the enzymes involved in these equilibria (results not shown). The calculated ATP/ADP ratio shows a substantial drop in the livers of the metformin-treated animals, values after treatment with 50 and 150 mg of metformin being 32 and 20 % of control values respectively. The data for oxaloacetate concentrations (83 and 82 %) shown in Figure 5 are also calculated values, based on the measured malate concentrations and the β-hydroxybutyrate/acetoacetate ratios [15].

DISCUSSION

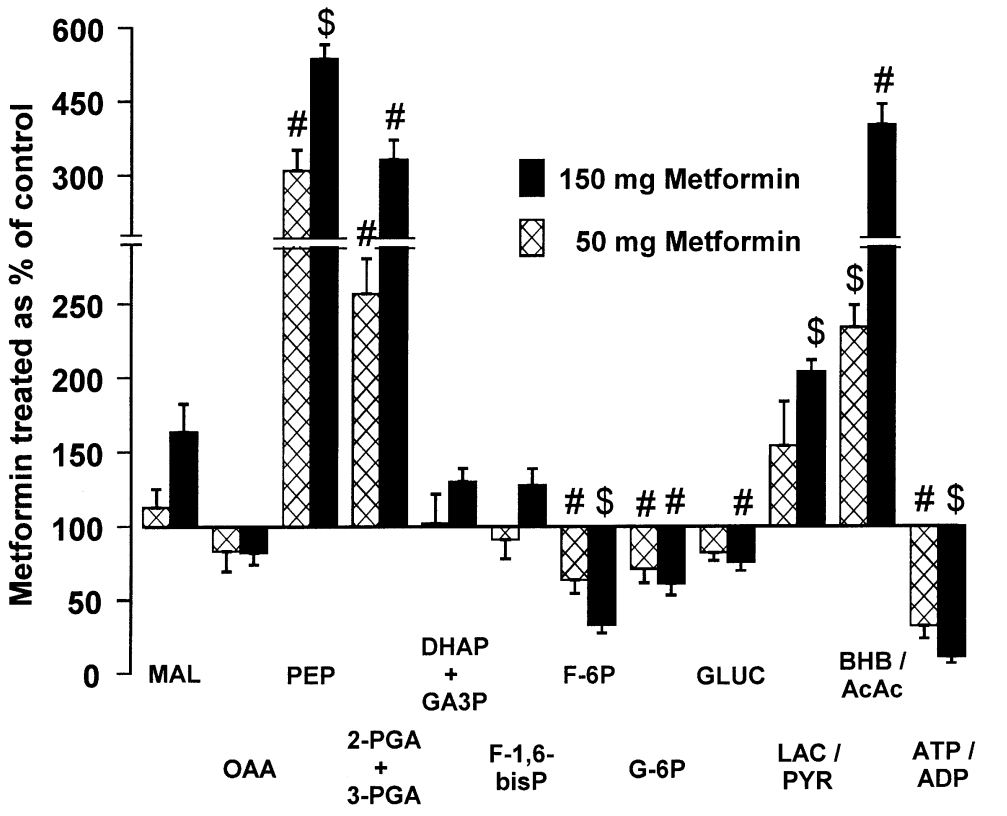
Metformin is slowly accumulated within mitochondria and directly inhibits complex 1

Comparison of the time and concentration dependence of complex-1 inhibition by metformin in mitochondria and SMPs suggest that there is a slow permeation of the drug across the mitochondrial inner membrane where it inhibits complex 1 directly. As would be predicted, phenformin, a more hydrophobic, lipid-soluble molecule, which binds more tightly to mitochondrial membranes [1–3], inhibited much more rapidly in intact mitochondria even at micromolar concentrations. The positive charge on metformin and phenformin could account for their accumulation within the matrix of energized mitochondria driven by the membrane potential ($\Delta\psi$). Such energy-dependent accumulation of biguanides within the matrix has been shown to occur experimentally [9] and is similar to that observed for some other positively charged hydrophobic inhibitors of complex 1 such as phenylpyridine analogues [32]. Indeed, the $K_{0.5}$ values for the inhibition of complex 1 by these compounds in energized mitochondria are 30–100-fold lower than with SMPs, a similar difference to that seen with phenformin in the present experiments (Figures 2b and 2c). A recent paper by El-Mir et al. [16] also demonstrated a specific time-dependent inhibition of complex 1 by 1–10 mM metformin in hepatocytes. These authors confirmed that these effects of metformin could not be demonstrated when the drug was added directly to mitochondria or permeabilized heptocytes, but was maintained after permeabilization of hepatocytes and when mitochondria were prepared from livers perfused with 10 mM metformin. However, they concluded from these data that the effect was indirect, through an unidentified signalling pathway. Our data suggest that there is no need to invoke such an indirect mechanism.

Inhibition of complex 1 by metformin can occur at drug concentrations found in treated patients

Thermodynamic considerations predict that with a $\Delta\psi$ of −180 mV, metformin should accumulate 1000-fold within the mitochondrial matrix. This would give a $K_{0.5}$ value with respect to extra-mitochondrial metformin of about 80 μM at equilibrium and is consistent with the 29 and 37 % inhibition of ADP-stimulated glutamate + malate oxidation observed in mitochondria from cultured rat hepatoma cells exposed for 60 h to 50 and 100 μM metformin respectively (Figure 1).

The plasma concentrations of metformin found in patients are about 10–20 μM [1–3]. However, the liver receives the majority of its blood via the portal vein, which may contain concentrations of metformin substantially higher than those present in the general circulation [25]. Thus significant inhibition of the respiratory chain of liver mitochondria in patients treated with metformin would be predicted. The observed inhibition of gluconeogenesis and pattern of hepatic metabolites in metformin-



Control metabolite concentrations (nmoles/mg protein) or ratios

MAL	2940 ± 150	DHAP + GA3P	49 ± 2	Glucose	26510 ± 2190
OAA	11.6 ± 1.0	F-1,6-bisP	20 ± 1	LAC/PYR	35.5 ± 3.8
PEP	75 ± 30	F-6P	158 ± 13	BHB/AcAc	0.28 ± 0.044
2/3-PGA	610 ± 140	G-6P	377 ± 57	ATP/ADP	2.60 ± 0.49

Figure 5 The effect of treatment of rats with metformin on the metabolite concentrations of their freeze-clamped livers

Treatment of rats with metformin (50 or 150 mg per rat) was performed daily for 5 days as described in the Experimental section. Control animals were given an equivalent volume of water. Metabolite concentrations for control livers ($n = 4$) are given in the table below; the values for ATP/ADP are the free cytosolic values calculated from the other metabolite levels as described in the text. The bar graph shows changes in metabolite concentrations and ratios induced by drug treatment ($n = 4$) expressed as percentages of the mean control values. The error bars represent the S.E.M. calculated from the ratios of the means and S.E.M. of the control and experimental data. The statistical significance of the effects of metformin were determined by an unpaired two-tailed Student's t test (#$P < 0.05$; \$$P < 0.01$). MAL, malate; OAA, oxaloacetate; PEP, phosphoenolpyruvate; 2- and 3-PGA, 2- and 3-phosphoglycerate; DHAP, dihydroxyacetone phosphate; GA3P, glyceraldehyde 3-phosphate; F-1,6-bisP, fructose 1,6-bisphosphate; G-6P, glucose 6-phosphate; F-6P, fructose 6-phosphate; GLUC, glucose; LAC/PYR, L-lactate/pyruvate; BHB/AcAc, β-hydroxybutyrate/acetoacetate.

treated rats provide further evidence that this is the case. Furthermore, in the case of phenformin, it has been shown by others that treatment of guinea pigs with 30 mg/kg of the drug leads to impaired oxidation of 2-oxoglutarate by subsequently isolated liver mitochondria [10]. We attempted similar experiments with control and metformin-treated rats (50 mg daily for 5 days) and determined rates of ADP-stimulated respiration by their liver mitochondria. To correct for non-specific effects on mitochondrial respiratory-chain activity, rates of respiration were expressed relative to the rate of ascorbate oxidation in the presence of N,N,N',N'-tetramethyl-p-phenylenediamine (which only utilizes cytochrome c and complex 4 of the respiratory chain). With glutamate + malate as respiratory substrate, the control and metformin ratios (expressed as means ± S.E.M. for five preparations of liver mitochondria from separate rats) were 0.86 ± 0.03 and 0.79 ± 0.09 respectively. In two rats treated with 150 mg for 5 days the ratios were 0.67 and 0.81. More substantial inhibition (about 50%) was observed by El-Mir et al. [16] after subcutaneous injection of rats with 600 mg/kg metformin or treatment of the perfused liver with 10 mM metformin for 30 min.

At first sight our own data may seem to indicate that little inhibition of complex 1 is occurring *in vivo* despite the changes in liver metabolite concentrations occurring that are characteristic of such inhibition. However, an alternative explanation is that the drug was lost from the mitochondria during their isolation, and previous data from this laboratory support this view. We have shown that during the standard isolation procedure, rat liver mitochondria become permeant to small hydrophilic compounds such as mannitol, which do not normally cross the inner membrane, even though they remain impermeant to sucrose [33]. In order to confirm that the permeability of the mitochondrial

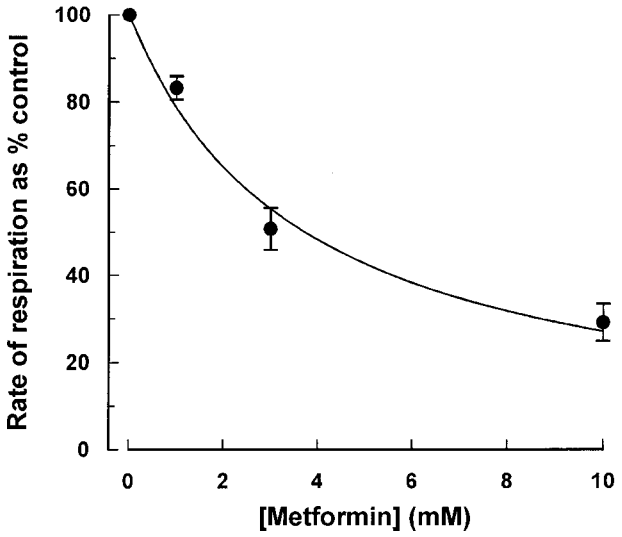


Figure 6 Isolation of liver mitochondria in the presence of metformin enhances the ability of the drug to inhibit NADH-dependent respiration

Rats livers were divided into four equal portions and each homogenized in standard sucrose isolation medium supplemented with 0, 1, 3 or 10 mM metformin as indicated. Isolation of mitochondria was performed as usual but metformin-supplemented buffers were used throughout. State-3 rates of respiration were then determined in the presence of glutamate + malate as substrate and metformin at the same concentration used for mitochondrial isolation. Each data point represents the mean ± S.E.M. of four separate mitochondrial preparations.

inner membrane to metformin also increases during mitochondrial isolation, rat livers were divided into four equal portions and each homogenized in normal sucrose media containing 0, 1, 3 or 10 mM metformin. Mitochondria were then isolated at 4 °C by the standard procedure using buffers supplemented with the same concentrations of metformin. This process takes about 90 min and yet the inhibition of glutamate + malate-dependent respiration by the metformin under these conditions (Figure 6) was considerably greater than that observed when isolated mitochondria were incubated at 8 °C for 4–5 h (Figure 2). This confirms that there is an increase in membrane permeability to metformin during mitochondrial preparation. Although the mechanism responsible is uncertain (see [33]) our data imply that a significant amount of the metformin accumulated *in vivo* will be lost during mitochondrial preparation. Thus the 10 % decrease in respiration rates that we observed following metformin treatment may reflect a substantially greater inhibition *in vivo*.

Inhibition of complex 1 by metformin can account for inhibition of gluconeogenesis

Our data demonstrate clearly that metformin produces a time- and concentration-dependent inhibition of gluconeogenesis in isolated hepatocytes, consistent with its progressive inhibition of the respiratory chain. It was not possible to reach a steady state of inhibition over the short time frame of these experiments, but longer incubations were not desirable since the control rates of gluconeogenesis could not be maintained. For this reason higher doses of metformin had to be used than occur *in vivo*. Nevertheless, the changes in intracellular metabolites seen in the hepatocytes (Figure 4) were the same as those determined in metformin-treated rats (Figure 5). They were also identical to those we have shown previously to accompany the action of other inhibitors of the respiratory chain and oxidative phosphorylation such as amytal, DCMU, oligomycin and atractyloside at sub-maximal doses [14,15]. These reagents also cause inhibition of gluconeogenesis without a significant decrease in total tissue ATP concentration, although decreases in the free ATP/ADP ratio do occur [15]. In these earlier experiments we performed a detailed analysis of the relationship between inhibition of complex 1 and inhibition of gluconeogenesis. We showed that 50 and 80 % inhibition of respiration led to 20 and 60 % inhibition of gluconeogenesis, respectively [14]. After a 30-min exposure of hepatocytes to 1 and 5 mM metformin, El-Mir et al. directly determined 25 and 60 % inhibition of complex 1 [16]. This would be predicted to give about 10 and 40 % inhibition of gluconeogenesis respectively, which is quite consistent with the inhibition we observed in our experiments (Figure 4). El-Mir et al. [16] also demonstrated increases in lactate/pyruvate and β-hydroxybutyrate/acetoacetate ratios in response to metformin, just as we did, but unlike us they detected a decrease in the ATP/ADP ratio determined from total adenine nucleotides. This probably reflects their use of 10 mM metformin as opposed to the 2 mM used in the present studies. Our previous work with respiratory-chain inhibitors demonstrated that decreases in both cytosolic and mitochondrial ATP/ADP ratios are observed as inhibition of the respiratory chain increases beyond 50 % [14,15].

Changes in the free ATP/ADP ratios cannot be measured directly since much of the ADP is believed to be bound and so masks significant changes in free [ADP], which is probably nearly two orders of magnitude less than the total [ADP] [34]. However, the calculated ATP/ADP ratio in the livers of metformin-treated rats was decreased substantially (Figure 5) and this is reflected in an increase in the 2-phosphoglycerate, 3-phosphoglycerate and phosphoenolpyruvate concentrations. We have demonstrated previously that the decrease in ATP/ADP ratio inhibits gluconeogenesis by inhibiting pyruvate carboxylase, an effect that may be reinforced by an increase in the cytosolic $NADH/NAD^+$ ratio and consequent decrease in cytosolic and mitochondrial pyruvate concentrations [14,15]. This is reflected by the modest decrease in calculated oxaloacetate concentrations (Figure 5 and [15]) and has been demonstrated directly by others using ^{13}C-tracer measurements [30]. In addition to an inhibition of pyruvate carboxylase, a stimulation of pyruvate kinase flux will occur as a result of the increased phosphoeneolpyruvate concentrations [14,15], an effect confirmed directly by others [27]. Together, these two effects will lead to an inhibition of gluconeogenesis, which may be further enhanced by the decrease in hexose phosphate concentrations leading to reduced flux through glucose-6-phosphatase.

Inhibition of complex 1 may explain other effects of metformin

Inhibition of complex 1 can also account directly for the modest inhibition of fatty acid oxidation that occurs following metformin treatment [1–3]. In addition, many other known effects of metformin treatment such as increased peripheral glucose metabolism and the stimulation of glycolytic lactate production by the intestine [1–3] would be predicted to occur as secondary consequences of respiratory-chain inhibition. The latter effect can be explained by a more extensive inhibition of mitochondrial respiration in the cells of the intestinal mucosa since these cells will be exposed to a higher local concentration of metformin in the intestinal lumen. The increase in insulin-dependent glucose utilization by peripheral tissues, including muscle and adipose tissue, that occurs following metformin treatment [2,3] is associated with increased expression of the plasma-membrane glucose transporters GLUT1 and GLUT4 [4–7]. It is known that

in many cells modest inhibition of the respiratory chain up-regulates the expression of glucose transporters and glycolytic enzymes [35,36] and causes glycolytic glucose utilization to be stimulated. Thus it would be predicted that modest inhibition of the respiratory chain by metformin would elicit the same response.

It might also be predicted that an increase in blood lactate would occur as a result of the increased glycolytic activity, and an increase is usually observed in patients treated with metformin [2,3,12,25,37,38]. The effect is usually small, probably reflecting the ability of other tissues such as heart and red muscle to oxidize excess lactic acid. However, an occasional side effect of metformin treatment is lactic acidosis [3], which would be predicted if inhibition of the respiratory chain by the drug treatment became excessive. This side effect is more common with phenformin [2,3,37,38], consistent with its more potent effects on the respiratory chain (Figure 2). Metformin probably avoids this complication because its inhibition may become self-limiting. As the matrix concentration of the drug increases, progressive inhibition of the respiratory chain will lead to a drop in membrane potential, which will prevent further accumulation of the drug.

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