Biochemical Pharmacology, Vol. 39, No. 11, pp. 1831–1834, 1990.
Printed in Great Britain.

0006-2952/90 $3.00 + 0.00

Sites of metformin-stimulated glucose metabolism

(*Received* 4 *December* 1989; *accepted* 6 *February* 1990)

The antihyperglycaemic agent metformin (dimethylbiguanide) is now widely used in the treatment of non-insulin-dependent diabetes mellitus (NIDDM) [1]. There is evidence that the drug lowers glucose concentrations by suppressing hepatic gluconeogenesis and enhancing peripheral glucose uptake. These actions of metformin require the presence of insulin, but the drug does not stimulate insulin release, suggesting a potentiation of insulin action, possibly by an effect at a postreceptor site of insulin action [1, 2].

The fate of glucose removed from the circulation by metformin remains uncertain. Therapeutic doses of metformin do not increase adiposity, but they can cause a small increase in blood lactate, mainly associated with meals [3–5]. However, the effects of metformin on aerobic and anaerobic glucose metabolism in different tissues have not been compared. This study investigates the effects of metformin on glucose oxidation (to CO_2) and anaerobic glucose metabolism (lactate production) by a range of tissues with different propensities for aerobic and anaerobic glucose metabolism using normal and streptozotocin (STZ) diabetic mice.

Materials and Methods

Adult male albino MF1 mice from a colony maintained at Aston University were used. The mice were housed in an air-conditioned froom at 22 ± 2° with a regular lighting schedule of 12 hr light (0800–2000 hr) and 12 hr dark. A standard pellet diet (Mouse Breeding Diet, Heygate & Sons Ltd, Northampton, U.K.) and tap water were provided *ad lib*. Diabetes was induced by intraperitoneal injection of streptozotocin (160 mg/kg, in citrate buffer pH 4.8) after an overnight fast. Only mice with a non-fasted plasma glucose concentration > 15 mmol/L after 1 week were accepted as diabetic.

Reagents of analytical grade and distilled water were used throughout. The chemicals and their sources were as follows: streptozotocin, crystalline bovine insulin (24.3 I.U./mg), bovine serum albumin (fraction V, RIA grade, insulin undetectable by radioimmunoassay), ethyleneglycol-bis beta-aminoethyl ether tetraacetic acid (EGTA) and collagenase (type IV) from the Sigma Chemical Co. (Poole, U.K); D-[U-^{14}C]glucose [sp. act. 270 mCi/mmol (10 GBq/mmol)] from Amersham International (Amersham, U.K.); sodium pentobarbitone (Sagatal) from May and Baker (Dagenham, U.K.); reagents for glucose analysis from Beckman Instruments Inc. (Galway, Ireland); lactate assay reagents from BCL (Lewes, U.K.); scintillant NE 260 from Nuclear Enterprises (Edinburgh, U.K.); pure metformin hydrochloride (batch 2452) from Lipha Pharmaceuticals (West Drayton, U.K.); other reagents from BDH (Poole, U.K.).

In vitro *studies*. Blood samples for plasma glucose [6] and insulin [7] were taken from the tail tip on the day before *in vitro* studies were conducted. Oxidative glucose metabolism was assessed by $^{14}CO_2$ production from [U-^{14}C]glucose and anaerobic glucose metabolism was assessed by lactate production using a range of tissues in which glucose uptake is acutely stimulated (diaphragm muscle and white fat) and not acutely stimulated (skin, liver, brain, renal medulla and intestine) by insulin. Fed mice were killed by cervical dislocation and the following tissues were rapidly excised: quarter diaphragms, pieces (approximately 20 mg) of epididymal fat and scrotal skin, and slices (approximately 20 mg) of brain and renal medulla. Overnight fasted mice were killed for removal of jejunum (proximal third of small intestine between the ligament of Treitz and the ileo-caecal junction) which was washed in saline and sliced into rings of approximately 20 mg. Tissue samples were individually preincubated for 15 min at 37° under an atmosphere of 95% O_2:5% CO_2 with gentle agitation (40 cycles/min) in Krebs–Ringer bicarbonate (KRB), pH 7.4, comprising in mmol/L 118 NaCl, 5 KCl, 25 $NaHCO_3$, 1.18 $MgSO_4$, 1.17 KH_2PO_4, 1.27 $CaCl_2$, saturated with 95% O_2:5% CO_2 and supplemented with bovine serum albumin (20 mg/mL), and glucose at the concentration (5, 10 or 25 mmol/L) used in the test incubation. Tissues from normal mice were incubated with 5 or 10 mmol/L glucose, whereas tissues from STZ diabetic mice were incubated with 25 mmol/L glucose to reflect the hyperglycaemia in these mice. Tissues were transferred into 25-mL vials for 2 hr test incubations at 37° with gentle agitation under an atmosphere of 95% O_2:5% CO_2. The vials contained 3 mL of the same medium as above, supplemented with D-[U-^{14}C]glucose (0.5 or 1.0 μCi/mL) without or with insulin and/or metformin. Insulin was added at a concentration of 10^{-8} mol/L for normal tissues and 10^{-6} mol/L for tissues of STZ diabetic mice due to the insulin resistance of peripheral tissues in STZ diabetes. Metformin was added at concentrations of 10^{-6} mol/L and 10^{-4} mol/L to each tissue, and also at 10^{-2} mol/L in jejunum which is known to accumulate metformin.

Hepatocytes were isolated from fed mice by the collagenase method used for rats [8]. Cell viability, assessed by 0.4% trypan blue exclusion, was accepted at > 90%. Test incubations were performed with a suspension of 7×10^6 viable cells/mL in a final volume of 1.5 mL/vial. Test buffer was the same as for tissue samples described above with 1 μCi/mL D-[U-^{14}C]glucose and unlabelled glucose (10 mmol/L), without and with insulin (10^{-8} mol/L) and metformin (10^{-6}, 10^{-4} and 10^{-2} mol/L).

Production of $^{14}CO_2$ was determined as previously [9]. Samples of clear supernatant obtained after centrifugation of the incubation medium were stored at −20° for lactate analysis [10].

In vivo *studies*. Groups of normal and STZ diabetic mice were fasted overnight. Metformin (250 mg/kg) in water (5 mL/kg) or water alone was administered by intragastric gavage 1 hr before administration of glucose (3 g/kg) by the same route. Anaesthesia was induced 50 min later by intraperitoneal injection of sodium pentobarbitone (60 mg/kg), and blood samples (50 μL) were collected 10 min later (1 hr after glucose administration) from the abdominal aorta before bifurcation of the iliac arteries, and from the inferior vena cava (IVC) below the entry of the renal veins, and the hepatic portal vein (HPV). The blood samples were withdrawn simultaneously through fine heparinized needles. Blood lactate [11] and plasma glucose [6] were determined.

Statistical analysis. Data are presented as mean ± SE. Groups of data were compared using Student's paired and unpaired *t*-tests as appropriate. Differences were considered to be significant if $P < 0.05$.

Results and Discussion

Compared with normal mice (N = 58), the STZ diabetic mice (N = 38) used for *in vitro* studies were typically hyperglycaemic (plasma glucose 33.6 ± 1.2 vs 8.1 ± 0.4 mmol/

L) and hypoinsulinaemic (plasma insulin 0.17 ± 0.07 vs 0.78 ± 0.05 ng/mL) with lower body weight (25.3 ± 0.8 vs 32.4 ± 0.3 g).

Diaphragm muscle of normal and STZ mice, incubated at 5 and 25 mmol/L glucose, respectively, showed no significant effect of metformin (10^{-6} and 10^{-4} mol/L) on aerobic and anaerobic glucose metabolism as assessed by CO_2 and lactate production in the absence and presence of added insulin (Fig. 1). Epididymal fat of normal mice also showed no significant effect of metformin alone on CO_2 or lactate production. However, in the presence of 10^{-8} mol/L insulin, 10^{-6} mol/L metformin increased (by 89%) CO_2 production by epididymal fat of normal mice without significantly affecting lactate production. The epididymal fat of STZ mice showed increased (by 129%) CO_2 production by metformin (10^{-4} mol/L) in the presence of insulin (10^{-6} mol/L), but there was no effect on lactate production or the production of CO_2 in the absence of added insulin.

Jejunal rings from fasted normal and STZ mice incubated at 10 mmol/L glucose showed no significant effect of metformin on CO_2 production. However, lactate production was significantly increased (about 20%) by metformin (10^{-2} and 10^{-4} mol/L in normal mice, and 10^{-2} mol/L in STZ mice) (Fig. 2).

At metformin concentrations of 10^{-6} and 10^{-4} mol/L, CO_2 and lactate production was not affected in hepatocytes, scrotal skin, brain and renal medulla (data not shown). However CO_2 production by hepatocytes of normal and STZ diabetic mice was decreased (by 39% in each case; $P < 0.05$) by 10^{-2} mol/L metformin in the presence of added insulin (10^{-8} mol/L), without a significant effect on lactate production (data not shown).

To further investigate the site at which postprandial plasma glucose is lowered and lactate is raised during metformin treatment, normal and streptozotocin diabetic mice were examined after intragastric administration of metformin (250 mg/kg at −2 hr) and glucose (3 g/kg at −1 hr). Simultaneous sampling at three sites (aorta, IVC, HPV) revealed that glucose was reduced (by about 25%) in blood of normal mice and (by 35–40%) in STZ diabetic mice (Table 1). However there were no consistent changes in the relative concentrations of glucose at the three sites in either normal or STZ diabetic mice. In contrast, blood lactate was raised (by 49–86%) in normal mice and (by 30–51%) in STZ diabetic mice. The greatest increase in blood lactate occurred within the HPV.

Treatment with metformin is known to increase glucose disposal in NIDDM as measured by intravenous glucose

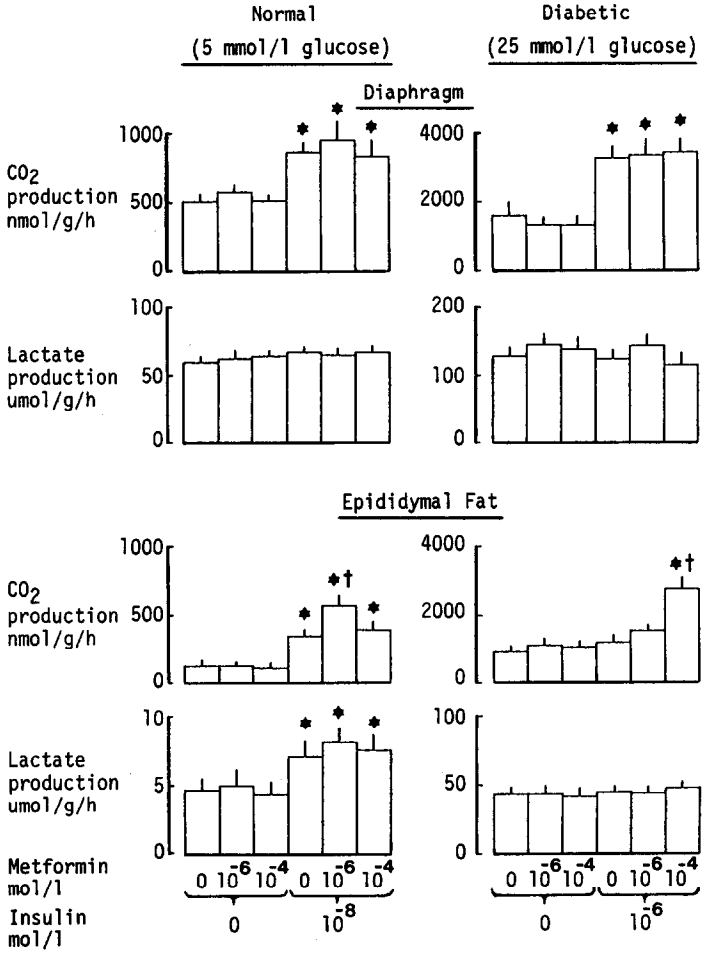

Fig. 1. Effect of metformin on aerobic (CO_2 production) and anaerobic (lactate production) glucose metabolism by diaphragm muscle and epididymal fat from fed normal and streptozotocin diabetic mice. Normal tissue was incubated with 5 mmol/L glucose and diabetic tissue with 25 mmol/L glucose. Insulin and metformin were added at the concentrations shown. Values are mean ± SE of 6–10 determinations. * $P < 0.05$ versus tissue without insulin but with same concentration of metformin; † $P < 0.05$ versus tissue with same insulin concentration but without metformin (Student's *t*-test).

Table 1. Effect of metformin (250 mg/kg at −2 hr) and glucose (3 g/kg at −1 hr) on plasma glucose and blood lactate concentrations in aorta, inferior vena cava (IVC) and hepatic portal vein (HPV) of normal and streptozotocin diabetic mice

	Plasma glucose (mmol/L)			Blood lactate (mmol/L)		
	Aorta	IVC	HPV	Aorta	IVC	HPV
Normal						
Control	12.9 ± 1.0	12.8 ± 1.0	13.0 ± 1.0	0.51 ± 0.07	0.40 ± 0.06	0.45 ± 0.07
Metformin	9.8 ± 0.9*	9.7 ± 0.9*	10.0 ± 0.8	0.76 ± 0.03*	0.66 ± 0.07*	0.84 ± 0.06†
Streptozotocin diabetic						
Control	26.6 ± 1.9	26.1 ± 1.9	26.6 ± 1.9	0.51 ± 0.07	0.40 ± 0.03	0.47 ± 0.04
Metformin	16.6 ± 1.5†	15.6 ± 2.1†	16.7 ± 2.0†	0.66 ± 0.07	0.57 ± 0.05*	0.71 ± 0.06*

The mice were fasted overnight and the metformin and glucose were given by intragastric gavage. Blood samples were collected under pentobarbitone anaesthesia. Values are mean ± SE of six mice.
* $P < 0.05$, † $P < 0.01$ versus control (Student's *t*-test).

tolerance and hyperinsulinaemic clamp techniques [12–14]. Metformin treatment lowered plasma glucose concentrations and improved insulin-stimulated glucose uptake and oxidation by soleus muscle of STZ diabetic mice [9], and incubation of diaphragms of alloxan diabetic rats with metformin increased glucose uptake in the presence of added insulin [15]. Glucose uptake and oxidation by normal human and rat adipose tissue was also enhanced during incubation with metformin [16–18]. The possibility of a direct effect of metformin on glucose metabolism by other tissues has not received attention.

The present study has examined a range of tissues from normal and STZ diabetic mice which use glucose as a major source of energy. These tissues show different acute responses to insulin and different propensities for aerobic and anaerobic metabolism. Of the tissues examined, namely diaphragm muscle, epididymal fat, hepatocytes, jejunum, skin, brain and renal medulla, only fat showed a direct stimulatory effect of metformin on aerobic glucose metabolism. This effect appeared to require the presence of insulin, consistent with the view that metformin potentiates insulin action. Evidence that metformin may improve insulin action by an effect on a postreceptor event has been presented elsewhere [1, 2, 19, 20]. Increased glucose oxidation by fat would be compatible with clinical evidence that the glucose-lowering effect of metformin, unlike that of sulphonylureas and insulin, is not associated with increased weight gain [4, 21]. Why metformin should directly enhance insulin-stimulated glucose oxidation by mouse fat, whereas muscle only showed a significant response after *in vivo* treatment [9] is uncertain. The *in vitro* procedure may not allow sufficient time for a measurable effect on muscle, or the effect on muscle may be at least partly a secondary effect due to the general improvement in glycaemic status after metformin treatment.

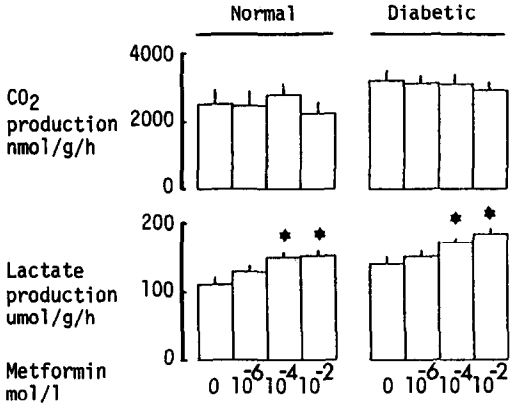

Fig. 2. Effect of metformin on aerobic (CO_2 production) and anaerobic (lactate production) glucose metabolism by rings of jejunum from overnight fasted normal and streptozotocin diabetic mice. Tissues were incubated with 10 mmol/L glucose and without added insulin. Values are mean ± SE of 11–16 determinations. * $P < 0.05$ compared with no metformin (Student's *t*-test).

The typical therapeutic range of plasma metformin concentrations is 10^{-6} to 5×10^{-5} mol/L. The highest plasma concentrations are found in the HPV. After an oral dose of 50 mg/kg metformin the plasma concentration in hepatic portal blood does not exceed 10^{-4} mol/L (authors' unpublished observations). A supratherapeutic concentration of metformin (10^{-2} mol/L) decreased glucose oxidation by hepatocytes, indicating a possible deleterious effect of this metformin concentration on hepatic glucose metabolism. Metformin is accumulated in jejunum [22], and studies in our laboratory have shown a maximum jejunal metformin accumulation of $> 10^{-3}$ mol/L tissue water 30 min after an oral dose of 50 mg/kg. This compares with a peripheral venous plasma metformin concentration of about 4×10^{-5} mol/L (authors' unpublished observations). Although metformin did not significantly alter glucose oxidation by jejunum of fasted mice, the drug increased lactate production by this tissue. Intestine shows a high propensity for anaerobic glucose metabolism (Fig. 2), and increased lactate production by the intestine could make a significant contribution to metformin-associated hyperlactataemia, particularly during absorption of a meal when the rate of glucose metabolism by the intestine is increased [23]. *In vivo* studies in which lactate was measured simultaneously in the aorta, IVC and HPV confirmed a net increase of lactate release into the portal vein after metformin treatment. There was no significant effect of metformin on net peripheral lactate production (i.e. no significant change in the arterio-venous lactate difference). This is consistent with the general lack of effect of metformin on lactate production by peripheral tissues *in vitro*, although this does not preclude the possibility that increased lactate production by certain peripheral tissues could be obscured by increased lactate extraction by other peripheral tissues.

It is noteworthy that metformin ($> 10^{-2}$ mol/L) enhanced lactate production from isolated rat hepatocytes when ethanol or glycerol were provided as substrate [24]. Other biguanides have been shown to increase glucose–lactate interconversion via the Cori cycle [25–27], but the effect of metformin on tissue lactate extraction has not been examined.

The present study indicates that the effect of metformin on glucose metabolism varies in different tissues. Several factors may be involved, such as the inherent bias of the tissue for aerobic and anaerobic metabolism, the insulin dependency of the tissue, and the concentration of metformin accumulated in the tissue. The effect of metformin on glucose metabolism is not merely the consequence of an effect on glucose uptake, since aerobic and anaerobic glucose metabolism can be affected differently in the same tissue. Metformin directly enhanced insulin-stimulated glucose oxidation by fat, which may bear relevance to the lack of weight gain accompanying metformin therapy. Metformin directly enhanced lactate production by the jejunum, which may make an important contribution to postprandial hyperlactataemia during metformin treatment.

Acknowledgements—The authors thank Dr Michael S. Billingham for his advice, and Nigel Coles for undertaking the blood lactate determinations. The excellent technical assistance of Susan L. Turner is gratefully acknowledged.

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