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EFFECTS OF METFORMIN ON GLUCOSE AND GLUCAGON REGULATED GLUCONEOGENESIS IN CULTURED NORMAL AND DIABETIC HEPATOCYTES

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Abstract—The effects of glucose and glucagon on the anti-gluconeogenic action of metformin were investigated in normal and diabetic hepatocytes. Glucose production from lactate was elevated by 88% in hepatocytes from fasted normal rats compared with hepatocytes from fed animals. Diabetes caused 3.5- and 2.1-fold increases in hepatic gluconeogenesis under fasting and fed conditions, respectively. Metformin (250 μM) suppressed glucose production by 37% in normal and by 30% in diabetic hepatocytes from fed rats. This drug was more effective (up to 67%) with increasing concentrations of glucose in the medium. Potentiation by metformin of insulin action on gluconeogenesis was elevated significantly ($P < 0.01$ to 0.001) by glucose *in vitro*. Metformin (75–250 μM) also counteracted the effects of glucagon at optimal concentrations in normal (32–68%) as well as diabetic (8–46%) hepatocytes. The findings of this study indicate that (i) the anti-gluconeogenic effect of metformin is enhanced by glucose *in vivo* and *in vitro*; and (ii) the suppression of glucagon-induced gluconeogenesis by metformin could play a role in its glucose-lowering effects in diabetic conditions.

Key words: metformin; diabetes; hepatocyte culture; gluconeogenesis; glucagon; insulin

Metformin, an oral antidiabetic drug, is widely used in the treatment of NIDDM† in Canada and Europe. This biguanide exerts its anti-hyperglycemic action by increasing peripheral glucose utilization and suppressing hepatic gluconeogenesis [1–7]. Metformin-induced inhibition of glucose production from various substrates has been demonstrated in isolated hepatocytes [7]. In NIDDM patients receiving metformin therapy, a significant reduction in hepatic glucose output has been observed [8].

Gluconeogenesis in the liver is regulated by the nutritional and hormonal status of the organism *in vivo* [9]. Glucagon plays a significant role in maintaining hepatic glucose output, whereas insulin suppresses this pathway [9]. Increased gluconeogenesis during starvation is due to changes in plasma levels of these hormones, as well as to decreased availability of glucose, which has a direct inhibitory effect on the expression of the key gluconeogenic enzyme, PEPCK [10]. Enhanced hepatic gluconeogenesis, an important factor in the development of hyperglycemia in diabetes, is the result of insulin deficiency and hyperglucagonemia [11]. Metformin has been shown to enhance the action of insulin and inhibit the effect of glucagon on gluconeogenesis in hepatocytes from normal rats [7, 12, 13]. However, there is limited information available, at present, on the interactions of metformin with hormones and glucose in the suppression of gluconeogenesis in diabetic hepatocytes.

The objective of this study was to evaluate the anti-gluconeogenic effect of metformin in cultured hepatocytes under conditions of elevated gluconeogenesis induced *in vivo* and *in vitro* by glucagon. We also examined the effects of glucose on the potentiation of the action of metformin in hepatocytes isolated from normal and STZ-induced diabetic rats.

MATERIALS AND METHODS

Materials. Dulbecco's modified Eagle's medium was purchased from GIBCO (Burlington, Canada). STZ and lactate were obtained from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). Collagenase was acquired from the Worthington Biochemical Corp. (Freehold, NJ, U.S.A.). Metformin was a gift of Nordic Laboratories Inc. (Kirkland, Canada). Crystalline glucagon and insulin were from Eli Lilly & Co. (Indianapolis, IN, U.S.A.). [^{125}I]Insulin (porcine) was purchased from Dupont Canada Inc. (Lachine, Quebec). [U-^{14}C]Lactate was obtained from ICN Biomedicals Inc. (St-Laurent, Canada).

Animals and cell culture. Male Sprague–Dawley rats (150–175 g) were made diabetic by a single intraperitoneal injection of STZ (60 mg/kg body wt) dissolved in 100 mM citrate (pH 4.5). Normal control rats were injected with the same volume of citrate buffer. The animals were fed *ad lib.* and kept under a constant 12-hr light–dark cycle. Diabetic rats with a non-fasting plasma glucose concentration of 17–22 mM were used 1 week after STZ injection. Hepatocytes were isolated from normal and diabetic

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† Abbreviations: NIDDM, non-insulin-dependent diabetes mellitus; STZ, streptozotocin; DMEM, Dulbecco's modified Eagle's medium; and PEPCK, phosphoenolpyruvate carboxy kinase.

rats in overnight fasted or fed condition by collagenase perfusion as described by Cascales *et al.* [14]. Cell viability, as assessed by the trypan blue exclusion test, was over 90%. The isolated hepatocytes were suspended in serum-free DMEM containing 0.1% bovine serum albumin. Three to four million cells were placed in 15 × 60 mm collagen-coated plastic dishes and cultured in a humidified atmosphere (5% CO_2) at 37° [15]. After 1 hr, unattached cells were removed, and the culture was continued with or without metformin for a subsequent period of 4 hr. Incubations for gluconeogenic studies were carried out using hepatocytes at this stage.

Measurement of gluconeogenesis. Gluconeogenesis was evaluated by measuring the amount of [^{14}C]-glucose formed from [U-^{14}C]lactate. The reactions were started by introducing gluconeogenic substrates, 5 mM [U-^{14}C]lactate (0.05 μCi/μmol) and 0.5 mM pyruvate along with different concentrations of glucose or glucagon. The incubation medium was collected after 0.5 or 1 hr for determination of [^{14}C]-glucose formed, and the cells were frozen in liquid nitrogen for DNA measurement. The radiolabeled glucose was separated from [U-^{14}C]lactate on an ion-exchange column consisting of 1 part Dowex 50W × 8 (H^+ form; 100–200 mesh) and 2 parts AG1 × 8 (acetate form; 100–200 mesh). The samples were applied to the column and were washed with distilled water [16]. [^{14}C]Glucose, which is not retained by the column, was collected in the wash eluant fractions. The rate of gluconeogenesis is expressed as micromoles of glucose produced from lactate per milligram of DNA per hour.

Analytical and statistical methods. Plasma glucose was measured by the glucose oxidase method using a diagnostic kit from Sigma. DNA content of hepatocytes was measured by the method described by Sorger and Germinario [17]. Statistical analysis was done by Student's *t*-test.

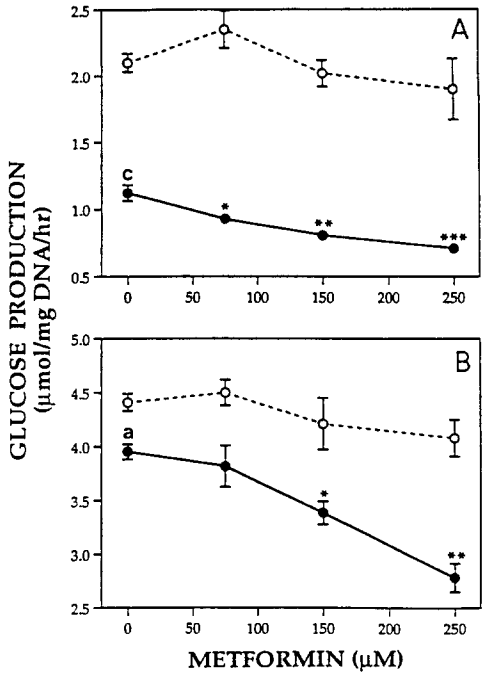

Fig. 1. Anti-gluconeogenic effect of metformin in cultured hepatocytes from fasted and fed rats. Hepatocytes were isolated from overnight fasted (—○—) and fed (—●—) rats and cultured in DMEM with 5.6 mM glucose. These cells were preincubated with various concentrations of metformin for 4 hr, and then the gluconeogenic substrates, 5 mM lactate (0.05 μCi/μmol) and 0.5 mM pyruvate, were added. The incubation medium was collected after 1 hr, and the [^{14}C]glucose formed was measured as described in Materials and Methods. (A) Normal hepatocytes; and (B) diabetic hepatocytes. Each point is the mean ±SEM of four independent experiments. All experiments were carried out in triplicate. Statistical significance: metformin-treated vs untreated cells: (*) $P < 0.05$; (**) $P < 0.01$; and (***) $P < 0.001$. Fasted vs fed hepatocytes: (a) $P < 0.05$; and (c) $P < 0.001$.

RESULTS

The effects of metformin on glucose production from lactate in cultured hepatocytes from normal and diabetic rats in fasted and fed conditions are shown in Fig. 1. In normal hepatocytes, the gluconeogenic rate was significantly higher in hepatocytes from overnight fasted liver than in hepatocytes from fed liver (2.1 ± 0.1 vs 1.1 ± 0.1 μmol/mg DNA/hr; Fig. 1A). Metformin (75–250 μM) had a concentration-dependent anti-gluconeogenic effect in hepatocytes from fed animals. Gluconeogenesis was decreased by 17% ($P < 0.05$) at a 75 μM concentration, and percent inhibition increased further with an increase in metformin concentrations (Fig. 1A). This effect was not apparent in cells from fasted rats. Glucose production from lactate was elevated significantly ($P < 0.001$) in diabetic hepatocytes in fasted as well as in fed conditions (Fig. 1B). Metformin (150–250 μM) decreased the gluconeogenesis by 14–30% in diabetic hepatocytes prepared from fed animals. No significant effect of metformin was observed in diabetic hepatocytes from fasted animals.

The effects of different concentrations of glucose on gluconeogenesis are shown in Fig. 2. The normal hepatocytes from fasted rats were sensitive to increasing glucose levels in the suppression of gluconeogenesis. After exposure to 10, 20 and 30 mM glucose, glucose production from lactate was 89, 78 and 61% of the control (at 5.6 mM glucose level), respectively (Fig. 2A). The anti-gluconeogenic effect of metformin was potentiated by increasing glucose concentrations. At 20 and 30 mM glucose, gluconeogenesis in metformin-treated normal hepatocytes was further decreased by 36 and 67%, respectively. In comparison with the normal cells, the untreated diabetic hepatocytes were less sensitive to the high glucose levels in the suppression of gluconeogenesis (Fig. 2B). In these diabetic cells, 10, 20 and 30 mM concentrations of glucose inhibited gluconeogenesis by 13, 19 and 20%, respectively. This inhibition was enhanced significantly ($P < 0.01$ to 0.001) by 20 and 30 mM glucose in the presence of metformin (250 μM).

The *in vitro* effect of glucose on the anti-gluconeogenic effects of metformin and insulin in

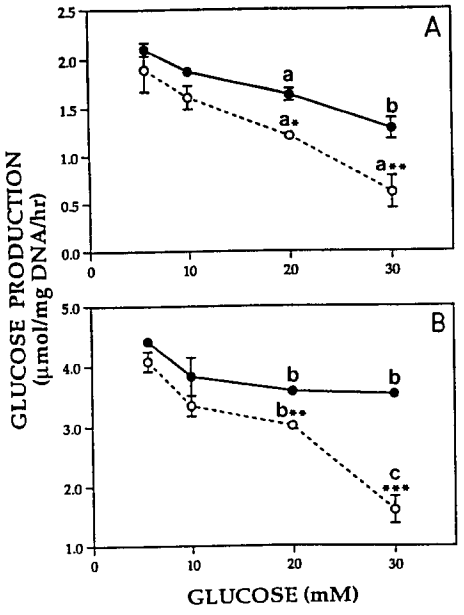

Fig. 2. Effect of glucose on the metformin-induced suppression of hepatic gluconeogenesis. Hepatocytes from overnight fasted normal (A) and diabetic (B) rats were preincubated in DMEM with different concentrations of glucose in the absence (—●—) and presence (—○—) of 250 μM metformin for 4 hr. Then, gluconeogenesis was measured as described in the legend of Fig. 1. Each point is the mean ± SEM of four independent experiments. All experiments were carried out in triplicate. Statistical significance: metformin-treated vs untreated cells: (*) $P < 0.05$; (**) $P < 0.01$; and (***) $P < 0.001$. Effects of high levels of glucose vs control (5.6 mM glucose) cells in their respective groups: (a) $P < 0.05$; (b) $P < 0.01$; and (c) $P < 0.001$.

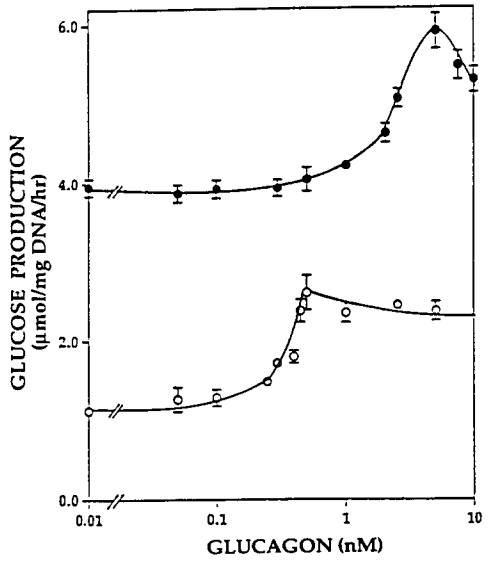

Fig. 3. Effect of various concentrations of glucagon on gluconeogenesis in normal and diabetic hepatocytes. Hepatocytes from normal (—○—) and diabetic (—●—) rats under the fed condition were incubated with increasing concentrations of glucagon. Gluconeogenesis from labeled lactate was measured as described in the legend of Fig. 1. Each point is the mean ± SEM of four independent experiments. All experiments were carried out in triplicate.

normal and diabetic hepatocytes are shown in Table 1. Metformin potentiated the inhibitory effect of insulin on glucose production by 27% in normal and by 18% in diabetic hepatocytes with a basal glucose concentration of 5.6 mM in the medium. This potentiation was enhanced further when the glucose concentration in the medium was raised to 30 mM. At this glucose concentration, metformin increased the anti-gluconeogenic effect of insulin by 61 and 64% in normal and diabetic hepatocytes, respectively.

Glucagon-stimulated gluconeogenesis from lactate in normal and diabetic hepatocytes from fed rats is shown in Fig. 3. Glucagon stimulated glucose production in a concentration-dependent manner in normal hepatocytes with the maximal increase at 0.5 nM. In the case of diabetic hepatocytes, this concentration–response curve was shifted to the

Table 1. Effects of metformin and glucose in the absence and presence of insulin on gluconeogenesis in normal and diabetic cultured rat hepatocytes*

Hepatocytes from	Glucose in the medium (mM)	No metformin	Plus metformin	Plus insulin	Plus insulin and metformin
Normal rats	5.6	2.02 ± 0.08	1.83 ± 0.25	2.21 ± 0.10	1.61 ± 0.13[a]
	30	1.29 ± 0.05†	0.79 ± 0.08†	1.27 ± 0.04‡	0.50 ± 0.04[c],†
Diabetic rats	5.6	4.41 ± 0.08	4.01 ± 0.13	3.63 ± 0.11	2.96 ± 0.17[a]
	30	3.56 ± 0.06†	1.61 ± 0.15‡	2.69 ± 0.12†	0.96 ± 0.08[c],‡

* Hepatocytes prepared from overnight fasted normal and diabetic rats were preincubated with and without metformin (250 μM) and/or insulin (10 nM) for 4 hr. The preincubation was in DMEM with 5.6 mM glucose at 37°. The procedure for the measurement of gluconeogenesis in the presence of 5.6 or 30 mM glucose was the same as that described in the legend of Fig. 1. Values are means ± SEM of four independent experiments. All experiments were carried out in triplicate.

†,‡ Glucose at 30 mM vs glucose at 5.6 mM in the respective groups: †$P < 0.01$, and ‡$P < 0.001$.

[a,c] Plus insulin vs plus insulin and metformin in respective groups: [a]$P < 0.05$, and [c]$P < 0.001$.

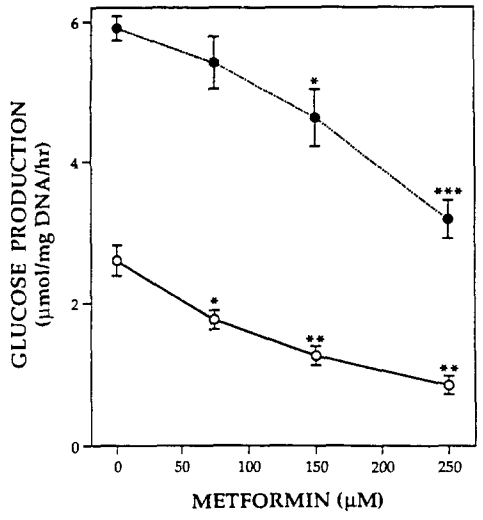

Fig. 4. Effect of metformin on glucagon-stimulated gluconeogenesis in normal and diabetic hepatocytes. Cultured hepatocytes from normal (—○—) and diabetic (—●—) rats were preincubated in the presence of various concentrations of metformin, and gluconeogenesis was stimulated with optimal concentrations of glucagon (0.5 nM-normal; 5 nM-diabetic). Glucose production from lactate was measured as described in the legend of Fig. 1. Each point is the mean ± SEM of four independent experiments. All experiments were carried out in triplicate. Statistical significance: metformin-treated vs untreated cells: (*) $P < 0.05$; (**) $P < 0.01$; and (***) $P < 0.001$.

right, the maximal stimulation being observed at 5 nM. The effects of metformin on glucagon-stimulated gluconeogenesis in normal and diabetic hepatocytes are shown in Fig. 4. Metformin (75–250 μM) had a concentration-dependent inhibitory effect on glucose production induced by optimal concentrations of glucagon in hepatocytes from normal and diabetic rats. Gluconeogenesis in normal liver cells in the presence of 0.5 nM glucagon was decreased by 32, 51 and 67% by 75, 150 and 250 μM metformin, respectively. In diabetic hepatocytes, metformin (150 and 250 μM) significantly decreased the glucagon-stimulated glucose production by 22 and 46%, respectively (Fig. 4).

DISCUSSION

In this study, we have shown that metformin can counteract the effects of glucagon on gluconeogenesis from lactate in cultured hepatocytes from normal and STZ-induced diabetic rats. This effect could play a significant role in the anti-hyperglycemic action of metformin in NIDDM patients since hyperglucagonemia is considered to be responsible for increased hepatic glucose output in these patients. The anti-gluconeogenic effect of metformin was further enhanced by glucose both *in vivo* and *in vitro*. These effects of metformin have been observed at concentrations close to therapeutic levels *in vivo* [18].

Gluconeogenesis was higher in hepatocytes from fasted rats than in hepatocytes from fed rats. This difference is due to the hormonal profile *in vivo* of these rats [9]. In fasted animals, due to the decrease in the plasma insulin level, glucagon-induced hepatic glucose production is elevated significantly. Upon feeding, the increased insulin secretion leads to suppression of this pathway. These hormones are known to regulate the activities of key gluconeogenic enzymes such as PEPCK. They have an antagonistic effect at the level of enzyme expression and have been shown to regulate the cytosolic protein binding to the 3′ end of PEPCK mRNA and hence the stability of PEPCK mRNA in cultured hepatocytes [19]. In diabetic hepatocytes, there was a significant increase in the rate of gluconeogenesis (Fig. 1). This elevation is due to insulin deficiency and hyperglucagonemia of the diabetic condition, leading to increased expression of gluconeogenic enzymes [9].

In both the fasted and fed conditions, in addition to changes in the plasma levels of glucagon and insulin, the availability of glucose also plays a role in the regulation of the gluconeogenic enzyme PEPCK. Glucose has been shown to decrease the expression of this enzyme by a dual mechanism [10]. It reduces the transcription of the PEPCK gene and accelerates the rate of PEPCK mRNA degradation. This effect is specific for glucose since other glucose-related carbohydrates did not alter the PEPCK mRNA levels. In the present study, glucose added in culture reduced glucose production from lactate in normal hepatocytes. However, the elevated gluconeogenesis in diabetic hepatocytes was suppressed only marginally by increasing the concentration of glucose in the medium (Fig. 2).

Metformin was not effective in suppressing glucose production from lactate in fasted hepatocytes from normal or diabetic rats. Under the fed condition, this biguanide significantly reduced gluconeogenesis; the effect was greater with diabetic hepatocytes. Hence, metformin seems to need the presence of glucose to exert its anti-gluconeogenic action. This drug is known to be an anti-hyperglycemic agent, and it does not reduce the glucose concentrations below euglycemia [1]. *In vitro* glucose was also able to enhance the effect of metformin. In normal and diabetic hepatocytes, with increasing concentrations of glucose in the medium, metformin decreased glucose production more efficiently (Fig. 2). The potentiation of insulin's action by metformin in normal and diabetic hepatocytes was greater with a higher glucose concentration in the medium (Table 1). Jackson *et al.* [8] showed that long-term treatment of NIDDM patients with metformin leads to decreases in basal hepatic glucose output and fasting blood glucose. A comparison of this study with our findings suggests that *in vivo* metformin can exert an anti-gluconeogenic effect under the fasting condition, probably by increasing the sensitivity to endogenous insulin in liver, whereas *in vitro* it does not decrease glucose production on its own in hepatocytes from fasted rats.

Glucagon increased the gluconeogenic rate in normal and diabetic hepatocytes *in vitro*. In diabetic cells, which show elevated gluconeogenesis, a higher glucagon concentration was needed to further

increase its rate (Fig. 3). In the presence of optimal concentrations of this hormone, metformin counteracted its effect on glucose production significantly in both normal and diabetic hepatocytes. Hence, metformin is effective in hepatocytes under conditions of elevated glucose production induced *in vitro* and *in vivo* by glucagon. In addition to having an opposite effect on glucose production, this biguanide is likely to counteract the hormone at the receptor level. The activation of adenyl cyclase by glucagon has been shown to be decreased by metformin [20]. This action can lead to a fall in the production of cyclic AMP and a decrease in the expression of the gluconeogenic enzyme PEPCK.

Metformin has been shown to increase peripheral and hepatic insulin sensitivity in NIDDM patients [8]. The ability of this hormone to inhibit adenyl cyclase in liver is restored in diabetic rats after metformin therapy [21]. In hepatocytes, metformin prevents insulin resistance [22] and potentiates its anti-gluconeogenic effect [7, 12]. It increases insulin-induced glucose uptake in adipocytes without affecting its receptor binding and tyrosine kinase activity [23]. However, metformin has been shown to increase insulin binding in a variety of cell types [24, 25]. Hence this anti-hyperglycemic agent is likely to enhance insulin action by acting at both the receptor and post-receptor levels. In the present study, metformin potentiated the inhibition of gluconeogenesis by insulin in normal and diabetic hepatocytes.

Metformin is known to exert its glucose-lowering effect by increasing peripheral glucose utilization and inhibiting hepatic glucose output. In addition to the potentiation of insulin's action, metformin also has other independent effects [1]. In muscle, metformin has been shown to translocate the glucose transporter from the intracellular pool to the plasma membrane [26]. The mechanism by which metformin inhibits hepatic gluconeogenesis is not clearly understood. Argaud *et al.* [27] showed that metformin decreases the concentration of cellular ATP, which is an allosteric inhibitor of pyruvate kinase. Hence, there is a stimulation of pyruvate kinase, which leads to inhibition of gluconeogenesis. Further studies are needed to examine the effects of metformin on the expression of the PEPCK gene.

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