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Effect of metformin on fatty acid and glucose metabolism in freshly isolated hepatocytes and on specific gene expression in cultured hepatocytes

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Abstract

The short-term effect of metformin on fatty acid and glucose metabolism was studied in freshly incubated hepatocytes from 24-hr starved rats. Metformin (5 or 50 mM) had no effect on oleate or octanoate oxidation rates (CO_2 + acid-soluble products), whatever the concentration used. Similarly, metformin had no effect on oleate esterification (triglycerides and phospholipid synthesis) regardless of whether the hepatocytes were isolated from starved (low esterification rates) or fed rats (high esterification rates). In contrast, metformin markedly reduced the rates of glucose production from lactate/pyruvate, alanine, dihydroxyacetone, and galactose. Using crossover plot experiments, it was shown that the main effect of metformin on hepatic gluconeogenesis was located upstream of the formation of dihydroxyacetone phosphate. Increasing the time of exposure to metformin (24 hr instead of 1 hr) led to significant changes in the expression of genes involved in glucose and fatty acid metabolism. Indeed, when hepatocytes were cultured in the presence of 50 to 500 μM metformin, the expression of genes encoding regulatory proteins of fatty acid oxidation (carnitine palmitoyltransferase I), ketogenesis (mitochondrial hydroxymethylglltaryl-CoA synthase), and gluconeogenesis (glucose 6-phosphatase, phosphoenolpyruvate carboxykinase) was decreased by 30 to 60%, whereas expression of genes encoding regulatory proteins involved in glycolysis (glucokinase and liver-type pyruvate kinase) was increased by 250%. In conclusion, this work suggests that metformin could reduce hepatic glucose production through short-term (metabolic) and long-term (genic) effects.

Keywords: Metformin; Fatty acid oxidation; Triglyceride synthesis; Gluconeogenesis; Crossover plot; Hepatic gene expression

1. Introduction

The antihyperglycemic agent metformin (dimethylbiguanide) has been used for over 40 years in the treatment of type 2 diabetes (reviewed in: [1]). Metformin, which does not stimulate insulin secretion (reviewed in: [2]), appears to act by improving the sensitivity of liver and peripheral tissues to insulin (reviewed in: [1,3]), although some controversy still remains (reviewed in: [3]). By contrast, it is now well admitted that the antihyperglycemic effect of metformin is due to the suppression of hepatic gluconeogenesis (reviewed in: [1,3]) either directly or as the result of an improved insulin action (reviewed in: [1,4]). However, the nature of the mechanism of metformin action on hepatic glucose production remains unclear. From *in vitro* studies, several explanations have been proposed for the reduction of hepatic gluconeogenesis: 1) a decrease in the uptake of gluconeogenic substrates by liver cells [5,6]; 2) an enhanced flux through pyruvate kinase (EC 2.7.1.40 ATP-pyruvate 2-*O*-phosphotransferase) [7] (reviewed in: [8]); and 3) a reduction in gluconeogenic flux through pyruvate carboxylase (EC 6.4.1.1 pyruvate:carbon-dioxyde ligase)/phosphoenolpyruvate carboxykinase (EC 4.1.1.38 diphosphate: oxaloacetate carboxy-lyase) [9]. Another aspect of the antihyperglycemic action of metformin is its ability to lower plasma free fatty acid and very-low-density lipoprotein-triglyceride (VLDL-TG) (reviewed in: [1,3,10]). The reduction in plasma free fatty acid (FFA) concentration following metformin treatment seems mainly due to a decrease in whole body FFA oxidation ([11], reviewed in: [10]). In-

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Abbreviations: CPT I or II, carnitine palmitoyltransferase I or II; mtHMG-CoA synthase, mitochondrial hydroxymethylglutaryl-CoA synthase; Glc-6-Pase, glucose-6-phosphatase; PEPCK, phosphoenolpyruvate carboxykinase; GK, glucokinase; L-PK, liver-type pyruvate kinase; and ACS, acyl-CoA synthetase.

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deed, accelerated FFA oxidation promotes hepatic gluconeogenesis by providing acetyl-CoA, ATP, and reducing equivalents (NADH, $FADH_2$; reviewed in: [12]) and reduces glucose utilization in peripheral tissues secondarily to an inhibition of pyruvate dehydrogenase activity (EC 1.2.1.51 pyruvate:NADP2 oxidoreductase (CoA-acetylation) (reviewed in: [13]). Therefore, a reduction in overall fatty acid oxidation by metformin would improve both hepatic glucose production and peripheral glucose oxidation. However, the effect of metformin on fatty acid oxidation is still a matter of debate. Indeed, if metformin reduces whole body FFA oxidation in some studies (see above), it fails to in others (reviewed in: [8]) or finally stimulates palmitoyl-CoA oxidation in isolated mitochondria [14]. Thus, it seems difficult to have a clear-cut idea concerning metformin action on fatty acid oxidation and its potential consequences on hepatic gluconeogenesis.

The aim of our study was to gain a better understanding of the effect of metformin on these two metabolic pathways in liver cell. This study was performed in hepatocytes isolated from 24-hr starved adult rats that exhibit active fatty acid oxidation and gluconeogenic rates. Moreover, very few studies have explored the effect of metformin on liver-specific gene expression. Thus, the effect of metformin on the expression of genes encoding regulatory proteins involved in glycolysis, gluconeogenesis, fatty acid oxidation, and ketogenesis has been determined in cultured hepatocytes isolated from 24-hr starved rats.

2. Materials and methods

2.1. Animals

Male Wistar rats (8–12 months old) weighing 200–300 g were housed in individual plastic cages and fed *ad lib*. standard laboratory food (68% carbohydrate, 11% fat, and 21% protein). In most of the experiments, rats were starved for 24 hr before hepatocyte isolation.

2.2. Isolation and incubation or culture of hepatocytes

Hepatocytes were isolated by *in situ* perfusion of the liver with 0.025% collagenase, as described previously [15]. Hepatocytes ($1–2 \times 10^6$ cells/mL) were incubated at 37° in 2 mL of oxygenated (O_2:CO_2; 95:5) Krebs–Henseleit bicarbonate buffer (pH 7.4) for 1 hr in a giratory shaking water bath. Each experiment was performed in duplicate. Metformin was dissolved in Krebs–Henseleit bicarbonate buffer and added to the incubation medium at a final concentration of 5 or 50 mM. For the study of gene expression in culture, hepatocytes were plated in 75-cm^2 Petri dishes ($3–5\ 10^6$ cells/dish) in an M199 glucose-free medium containing penicillin (10 UI/mL), streptomycin (100 μg/mL), and kanamycin (50 μg/mL). During cell attachment (4 hr), a substitute of fetal bovine serum (Ultroser G, 2%; IBF) was present. Duplicate dishes were used for all experiments. The cultures were maintained for 24 or 48 hr at 37° in an incubator equilibrated with O_2/CO_2 (95/5%). Metformin was used at a final concentration of 50, 100, or 500 μM.

2.3. Measurement of fatty acid oxidation and esterification

Long- and medium-chain fatty acid metabolism was studied using [l-^{14}C]oleate (0.3 mmol/L; 0.5 μCi/μmol) plus carnitine (0.5 mmol/L) and [l-^{14}C]octanoate (0.6 mmol/L, 0.1 μCi/μmol), respectively. Both fatty acids were bound to 2% (w/v) defatted albumin. Incubations were ended by adding 0.2 mL of perchloric acid ($HClO_4$ 40% v/v). The production of $^{14}CO_2$ and labelled acid-soluble products was determined as described previously for [^{14}C]oleate [16] and [^{14}C]octanoate [17]. For studies of oleate esterification, incubations were ended by centrifugation for 30 sec at 3000 g. The lipids from chloroform/methanol (2/1, v/v) cell-pellet extracts were separated by thin-layer chromatography, as previously described [18].

2.4. Glucose production rates and gluconeogenic intermediate concentrations

The rates of gluconeogenesis were determined after a 1-hr incubation period in the absence (endogenous) or in the presence of lactate/pyruvate (10/l mM), or alanine (10 mM), or dihydroxyacetone (10 mM) or galactose (10 mM). Gluconeogenic intermediate concentrations were measured in hepatocytes incubated for 1 hr in the presence of alanine (10 mmol/L) either in the absence of metformin (control) or in the presence of 5 mM metformin. The incubations were ended by adding 0.2 mL $HClO_4$ (40% v/v).

2.5. Metabolite analysis

Ketones, gluconeogenic intermediates, ATP, and ADP concentrations were measured in the neutralized perchoric filtrates by enzymatic methods as described previously [19]. Oxaloacetate was calculated according to the following formula:

$$[\text{oxaloacetate}] = \frac{[\text{pyruvate}] \times [\text{malate}] \times k_{\text{MDH}}}{[\text{lactate}] \times k_{\text{LDH}}}$$

where k_{MDH} and k_{LDH} represent the equilibrium constants of malate dehydrogenase (2.78×10^{-5}, EC 1.1.1.37 (*S*)-malate:NAD oxidoreductase) and lactate dehydrogenase (1.1×10^{-4}, EC 1.1.1.27 (*S*)-lactate:NAD oxidoreductase) respectively.

2.6. Extraction and Northern blot analysis of total RNA

Total RNA from cultured hepatocytes of two Petri dishes were extracted using the one-step technique according to

Table 1
Effect of metformin on oleate and octanoate metabolism in isolated hepatocytes from 24-hr starved rats

	Control	Metformin 5 mM	Metformin 50 mM
Oleate [l-^{14}C]			
CO_2	8.4 ± 1.3	7.3 ± 1.1	6.0 ± 0.6
Acid-soluble Products	77 ± 10	78 ± 11	61 ± 12
Acetoacetate	144 ± 9	98 ± 8	27 ± 3**
β-Hydroxybutyrate	104 ± 11	166 ± 66*	200 ± 19**
B/A	0.7 ± 0.1	1.7 ± 0.2*	7.4 ± 0.1**
Octanoate [l-^{14}C]			
CO_2	10.4 ± 1.6	7.9 ± 1.1	10.6 ± 1.5
Acid-soluble Products	161 ± 15	147 ± 8	163 ± 16
Acetoacetate	172 ± 7	122 ± 8*	46 ± 2**
β-Hydroxybutyrate	223 ± 52	305 ± 47*	280 ± 16
B/A	1.3 ± 0.2	2.5 ± 0.1*	6.1 ± 0.1**

Hepatocytes were incubated for 1 hr in the absence or in the presence of metformin at the indicated concentration. The rates of fatty acid oxidation and ketogenesis were determined from [l-^{14}C]oleate (0.3 mM) or [l-^{14}C]octanoate (0.6 mM) bound to 2% fat-free albumin. Results are expressed as nmol/hr/10^6 hepatocytes and are means ± SEM of 6 to 10 different experiments performed in duplicate.
* $P < 0.05$ and ** $P < 0.01$ when compared to control.

[20]. RNA was quantified by ultraviolet absorbance at 260 nm (260/280 ratio > 1.8) and 1 μg was submitted to electrophoresis in 1% agarose gel to check the quality of the RNA preparation. Northern blot analysis of total RNA (20 μg) was performed after 1% agarose gel electrophoresis in 2.2 M formaldehyde as previously described [21]. Hybridization of the blots with an excess of [γ-^{32}P]ATP-labelled synthetic oligonucleotide specific for the 18S rRNA subunit [22] allowed us to correct for possible variations in the amount of RNA transferred onto the membranes. The hybridization probes used were: the 0.9-kb *Eco*R1-*Bam*H1 from pGEM4-Glut-2 [23], the 1.8-kb fragment from pUC-GK1 [24], the 1.1-kb *Pst*-1 fragment for Glc-6-Pase [25], the 2.6-kb *Pst*-1 fragment from PCK 10 [26], the 530-bp *Eco*RV fragment from ACS cDNA [27], the *Eco*R1 fragment from p61a CPT I [28] and from pBKS-CPT II 4 [29], the *Kpn*1 fragment from pMS1-HMG-CoA synthase [30]. Probes were radiolabeled using the multiprime DNA labeling system (Amersham). Quantifications were performed by scanning densitometry of the autoradiographs.

2.7. Statistical analysis

Results are expressed as means ± SEM. Statistical analysis was performed using the rank-order test [31].

3. Results

The first goal of this work was to look at the acute effect of metformin on glucose and fatty acid metabolism in 1-hr incubated hepatocytes. As previously shown [7], this experimental model required a high concentration of metformin because of the short time period of exposure to the drug.

3.1. Effect of metformin on fatty acid metabolism in incubated hepatocytes

3.1.1. Fatty acid oxidation

The rates of long-chain (oleate) or medium-chain (octanoate) fatty acid oxidation were estimated by measuring the production of radiolabeled CO_2 and acid-soluble products (ASP, Krebs cycle intermediates and ketones). As shown in Table 1, metformin (5 or 50 mM) did not affect oleate or octanoate oxidation rates (Table 1). Similarly, metformin did not affect the rate of total ketone body production (acetoacetate + β-hydroxybutyrate, Table 1). This absence of effect of metformin on fatty acid oxidation was specific and not due to a low sensitivity of incubated hepatocytes, since metformin markedly affected the proportion of each ketone body produced. Metformin inhibited in a concentration-dependent manner the production of acetoacetate, whereas it stimulated the production of β-hydroxybutyrate, whether oleate or octanoate was used (Table 1). Such opposite effects on ketones led to a concentration-dependent increase in the β-hydroxybutyrate to acetoacetate ratio (B/A, Table 1) which is commonly used as an index of the intramitochondrial redox state (NADH/NAD^+ ratio).

3.1.2. Fatty acid esterification

Since octanoate cannot be incorporated directly into cell triglycerides, only the rate of oleate esterification was investigated. As the rates of fatty acid esterification are relatively low in hepatocytes from fasting rats (15 to 20% of total oleate metabolized), we also investigated the effect of metformin in cells that have a greater capacity for fatty acid esterification, i.e. hepatocytes from fed adult rats. However, whatever the source of hepatocytes (fed versus starved) or the concentration of metformin (5 or 50 mM) used, it had no effect on triglyceride and phospholipid synthesis (Table 2).

Table 2
Effects of metformin on oleate esterification in hepatocytes isolated from fed or 24-hr starved rats

	Control	Metformin 5 mM	Metformin 50 mM
24-hr-starved			
Triglycerides	11.5 ± 0.9	9.1 ± 0.7	12.9 ± 2.2
Phospholipids	3.7 ± 0.4	2.9 ± 0.4	5.6 ± 1.0
Fed			
Triglycerides	69.4 ± 4.9	70.2 ± 5	66.0 ± 4.2
Phospholipids	9.8 ± 0.5	9.8 ± 0.5	9.7 ± 0.5

Hepatocytes were incubated for 1 hr in the presence of [l-^{14}C]oleate (0.3 mM) bound to 2% fat-free albumin and either in the absence or in the presence of metformin at the indicated concentration. Results are expressed as nmol/hr/10^6 hepatocytes and are means ± SEM of 6 to 8 different experiments performed in duplicate.

3.2. Effect of metformin on hepatic gluconeogenesis in incubated hepatocytes

As shown in Fig. 1, metformin inhibited in a concentration-dependent manner the rates of glucose production, whatever the gluconeogenic substrates used. To localize the putative(s) step(s) affected by metformin, we used the cross-over plot technique from alanine which determines all the metabolic intermediates of gluconeogenesis. Crossover plot analysis showed that metformin inhibited glucose production mainly upstream of dihydroxyacetone phosphate synthesis (Fig. 2). Metformin (5 mM) induced similar changes in the cytosolic NADH/NAD^+ ratio as estimated by the 3-fold increase in the lactate/pyruvate ratio (respectively 9.6 ± 0.3 versus 3.5 ± 0.2 for control, N = 9, $P < 0.01$). In addition, metformin (5 mM) induced a 60% decrease in the ATP/ADP ratio (respectively 3.6 ± 0.6 for control versus 1.3 ± 0.4 for metformin-treated cells N = 5, $P < 0.05$). This resulted from a 70% lower ATP concentration in metformin-incubated hepatocytes (3.0 ± 0.4 nmol/10^6 hepatocytes) than in the control hepatocytes (11.2 ± 1.0 nmol/10^6 hepatocytes), although the ADP concentrations were not affected by metformin (3.2 ± 0.8 and 3.5 ± 0.4 nmol/10^6 hepatocytes for control and metformin respectively).

The second goal of this work was to analyse whether metformin could have a long-term effect on the expression of gene encoding regulatory proteins involved in glucose and fatty acid metabolism. As previously reported in several studies (reviewed in: [4]), increasing the length of exposure to metformin, made it possible to decrease the concentration used. Thus, experiments were carried out in hepatocytes cultured for 24 or 48 hr in the presence of 50, 100, or 500 μM of metformin.

3.3. Effect metformin on hepatic gene expression in cultured hepatocytes

As the maximal effects of metformin were already obtained after 24 hr of culture, only these results will be presented. Two classes of genes were studied: those coding for regulatory proteins of glucose metabolism and those encoding key enzymes of mitochondrial fatty acid metabolism. Although metformin had no effect on hepatic glucose transporter Glut-2 (Table 3), it had opposite effects on glycolytic and gluconeogenic enzyme gene expression. Both glucokinase (EC 2.7.1.2. ATP-D-glucose6 phosphotransferase) and L-pyruvate kinase (EC 2.7.1.40 ATP-pyruvate 2-*O*-phosphotransferase) mRNA levels were increased by the highest concentration of metformin (500 μM) used

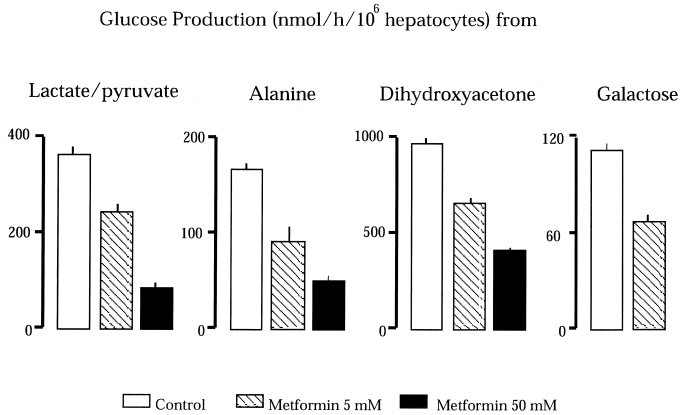

Fig. 1. Dose-dependent effect of metformin on hepatic glucose production from lactate/pyruvate (10/l mM), alanine (10 mM), dihydroxyacetone (10 mM), or galactose (10 mM). Hepatocytes were incubated for 1 hr in the absence (control) or in the presence of metformin at the indicated concentration. Endogenous glucose production (no substrate added for 1 hr) was subtracted from each value. Results are means ± SEM of 5 to 10 different experiments. The effects of metformin were statistically different from control for $P < 0.01$ whatever the concentration tested.

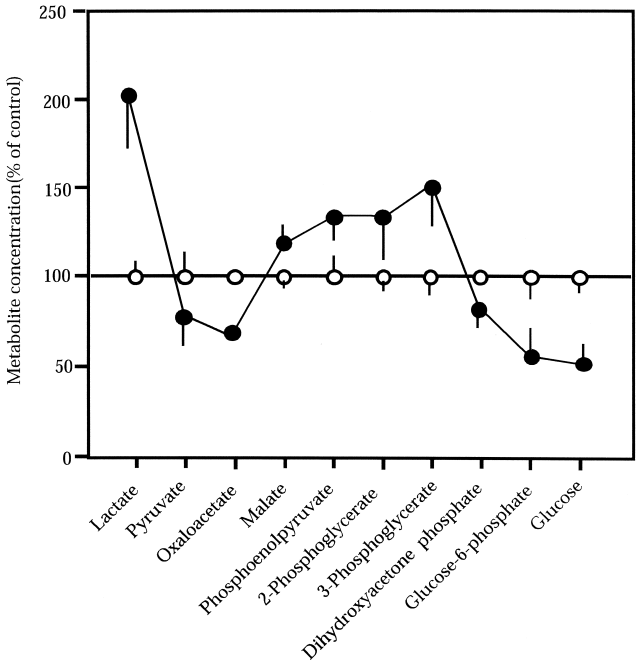

Fig. 2. Crossover plot showing the effect of metformin on the concentration of gluconeogenic intermediates in isolated hepatocytes incubated for 1 hr in the presence of alanine 10 mM. The metabolite concentration in 1 hr metformin-treated hepatocytes (5 mM ●) was expressed as a percent of that found in control hepatocytes (○). The concentration of each metabolite in control cells is given in nmol per 10^6 hepatocytes: lactate = 55 ± 8; pyruvate = 17 ± 3; calculated oxaloacetate = 0.3; malate = 3.5 ± 0.4; phosphoenolpyruvate = 2.8 ± 0.5; 2-phosphoglycerate = 0.8 ± 0.2; 3-phosphoglycerate = 4.1 ± 0.8; dihydroxyacetone phosphate = 4.1 ± 0.7; glucose-6-phosphate = 0.50 ± 0.09; glucose = 167 ± 19. Results are means ± SEM of 9 different experiments performed in duplicate.

(Table 3), whereas for similar concentrations it reduced the expression of Glc-6-Pase (EC 3.1.3.9 D-glucose-6-phosphate phosphohydrolase) and PEPCK genes (Table 3). It should be noted that the effect on GK and PEPCK mRNA levels was already perceptible at a metformin concentration of 100 μM (Table 3). Concerning the expression of genes involved in mitochondrial fatty acid metabolism, metformin had no effect on ACS (EC 6.2.1.3 acid CoA ligase) and CPT II (EC 2.3.1.21 palmitoyl-CoA L-carnitine O-palmitoyltransferase) mRNA levels, but decreased the expression of CPT I and mtHMG-CoA synthase (EC 4.1.3.5 3-hydroxy-3-methyl-CoA acetoacetyl-CoA lyase) (Table 3), the rate-limiting enzymes in mitochondrial long-chain fatty acid oxidation and ketogenesis, respectively.

4. Discussion

Before considering the effect of metformin *in vitro*, we must emphasize that the concentrations of metformin used in incubated or cultured hepatocytes are much higher than those observed in human therapy (10 to 50 nM, reviewed in: [4]). Despite the fact that most clinical studies have shown that the main action of metformin was to decrease hepatic glucose output secondarily to an inhibition of gluconeogenesis (reviewed in: [4,32]), the above statement means that we must keep in mind that the incubated or cultured hepatocyte represent experimental models used to dissect metformin action.

The first goal of the present work was to study the putative effect of metformin on hepatic fatty acid metabolism. Neither fatty acid oxidation nor triglyceride synthesis was affected by metformin whatever the concentration used

Table 3
Dose-dependent effect of metformin on mRNA levels of genes encoding regulatory proteins of glucose and fatty acid metabolism in cultured hepatocytes from 24-hr starved rats

Hepatic genes	% of control value		
	Met 50 μM N = 5	Met 100 μM N = 6	Met 500 μM N = 8
Glut-2	102 ± 16	96 ± 3	110 ± 23
Glycolysis/Gluconeogenesis			
Glucokinase (GK)	112 ± 20	140 ± 26	250 ± 34**
Glucose-6-phosphatase (Glc-6-Pase)	109 ± 15	ND	72 ± 11*
L-Pyruvate kinase (L-PK)	124 ± 12	ND	250 ± 50*
Phosphoenolpyruvate carboxykinase (PEPCK)	120 ± 20	85 ± 8	65 ± 7*
Fatty acid oxidation/Ketogenesis			
Acyl-CoA synthetase (ACS)	128 ± 20	110 ± 20	103 ± 23
Carnitine palmitoyltransferase I (CPT I)	120 ± 8	95 ± 10	63 ± 8*
CPT II	104 ± 10	102 ± 5	106 ± 9
Hydroxymethylglutaryl-CoA synthase (mtHMG-CoA Synthase)	110 ± 18	87 ± 24	55 ± 10**

Total RNA were extracted from 24-hr-starved adult rat hepatocytes cultured in the absence or in the presence of metformin at the indicated concentrations. Results are means ± SEM and are expressed as percentage of mRNA levels found in hepatocytes cultured in control conditions. ND = not determined.
*$P < 0.05$ and **$P < 0.01$ when compared to control.

(5 or 50 mM). This did not result from the experimental model used, since under similar conditions of incubation of 24-hr starved rat hepatocytes, thiazolidinediones markedly reduced oleate oxidation and esterification [33]. These two metabolic pathways will be discussed separately.

Although metformin did not affect the rates of fatty acid oxidation or ketone body production, it did induce a marked rise in the β-hydroxybutyrate/acetoacetate ratio and thus a more reduced mitochondrial redox state. This effect on the $NADH/NAD^+$ ratio results from an inhibition of the respiratory chain complex I [34,35]. This inhibitory effect on the respiratory chain affects whole energy metabolism in intact hepatocytes, especially the ATP status whose concentration was decreased in metformin-treated hepatocytes [7,34], present work). The fact that metformin failed to decrease hepatic fatty acid oxidation in short-term exposed hepatocytes differs from data obtained in obese and non-obese NIDDM patients in whom metformin treatment was associated with a reduction in both whole body FFA oxidation and plasma β-hydroxybutyrate concentration [11]. These discrepancies could be explained by several non-exclusive ways: 1) the decrease (−25%) in overall lipid oxidation and the associated fall in plasma β-hydroxybutyrate concentration could be mainly due to a decrease in plasma FFA availability in metformin-treated patients [10,11]; 2) the inhibitory effect of metformin on whole-body lipid oxidation was observed during a mild-hyperinsulinemic-isoglycemic clamp [11]; 3) finally, another possibility for the differences between these two studies could be due to the length of exposure to metformin, several hours in Perriello's work versus 1 hr in our work. In keeping with this, we showed that increasing the length of exposure to metformin induced a modest but significant decrease in the level of mRNA encoding CPT I and mtHMG-CoA synthase, the rate-limiting enzymes of mitochondrial long-chain fatty acid oxidation and ketogenesis, respectively. Unfortunately, the delay between changes in mRNA level and changes in protein did not allow metabolic studies since after 48 hr of culture, hepatocytes lost most of their differentiated functions, especially fatty acid oxidation and ketogenesis (data not shown). However, if we assume that changes in CPT I and mtHMG-CoA synthase protein levels parallel those of their respective mRNA levels, this would suggest that metformin could slightly reduce hepatic fatty acid oxidation and ketone body production during long-term treatment, at least in the rat. Whether this long-term effect of metformin is responsible for reduced hepatic gluconeogenesis remains to be determined. What does seem clear is that metformin cannot be considered as a potent inhibitor of hepatic fatty acid oxidation as shown, for example, for troglitazone [33].

Another interesting aspect of metformin action is its lowering VLDL-triglyceride effect (reviewed in: [1,3,10]). This effect is due to the combination of a decreased synthesis and increased clearance of VLDL (reviewed in: [1]). However, there is, to our knowledge, no study demonstrating a direct effect of metformin on liver capacity for triglyceride synthesis, i.e. fatty acid esterification. Indeed, the present work demonstrates that metformin did not affect the capacity for fatty acid esterification, regardless of whether this metabolic pathway was fully active (hepatocytes from fed rats) or reduced (hepatocytes from fasting rats). This suggests that decreased hepatic VLDL-triglyceride synthesis is probably due to the reduced availability in plasma FFA, secondarily to the antilipolytic effects of metformin on adipose tissue [36,37].

The original goal of this work was to look at the putative effects of metformin on fatty acid oxidation–gluconeogenic relationships. As discussed above, it is clear that, at least during short-term exposure, the inhibitory effect of metformin on hepatic gluconeogenesis is not mediated by a decreased rate of fatty acid oxidation. This was confirmed: 1) by a crossover study showing that none of the specific steps (pyruvate carboxylase, glyceraldehyde-3-phosphate dehydrogenase) controlled by fatty acid oxidation-derived cofactors (acetyl-CoA and NADH) are affected by metformin treatment; and 2) by an inhibitory effect of metformin on hepatic gluconeogenesis whatever the substrate used, as previously demonstrated in a similar experimental model [5,7,38]. The fact that glucose production from galactose was also inhibited by metformin suggests that its effect on hepatic gluconeogenesis was not restricted to a "control point" above dihydroxyacetone phosphate as previously discussed (review in: [8]), but could also alter the glucose-6-phosphatase/glucokinase enzyme cycle. Indeed, in rats fed a high-fat diet, it was reported that metformin treatment increased the ratio glucose-6-phosphatase/glucokinase activities mainly as the result of a decrease in the activity of hepatic glucose-6-phosphatase [39]. Our work suggests that, in addition to its effect on enzyme activity, metformin induced a decrease in glucose-6-phosphatase gene expression and a stimulation of glucokinase gene expression at least *in vitro*. If such modifications in gene expression were associated with corresponding changes in enzyme activity, metformin would have anti-hyperglycemic effects through a combined stimulation of glycolysis and inhibition of gluconeogenesis. Moreover, our study provides evidence that metformin also affects the expression of genes encoding regulatory proteins of the phosphoenolpyruvate/pyruvate cycle. Indeed, metformin enhanced the expression of L-pyruvate kinase, whereas it decreased PEPCK gene expression. By contrast, metformin did not affect the expression of the glucose transporter Glut-2, as previously shown in the small intestine of metformin-treated rats [40]. These results indicate that metformin can regulate the expression of specific hepatic genes in an insulin-independent manner. Interestingly, similar observations were observed with troglitazone, another oral anti-diabetic agent [41]. Although the molecular basis for the action of metformin on gene expression remains to be determined, this could be in relation to its effect on cellular redox state. Indeed, it was reported that redox status of the cell is involved in the control of numerous genes (reviewed in: [42]).

In conclusion, this work suggests that the antihyperglycemic action of metformin could be due, at least in part, to the reduction of hepatic gluconeogenesis through short-term (metabolic) and long-term (gene expression) effects. Although metformin has no acute effect on hepatic fatty acid metabolism, its inhibitory effect on CPT I gene expression could also participate in its lasting lowering action on hepatic gluconeogenesis.

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