Effects of Benfluorex on Fatty Acid and Glucose Metabolism in Isolated Rat Hepatocytes

From Metabolic Fluxes to Gene Expression

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The effects of benfluorex and two of its metabolites (S 422-1 and S 1475-1) on fatty acid and glucose metabolic fluxes and specific gene expression were studied in hepatocytes isolated from 24-h fasted rats. Both benfluorex and S 422-1 (0.1 or 1 mmol/l) reduced β-oxidation rates and ketogenesis, whereas S 1475-1 had no effect. At the same concentration, benfluorex and S 422-1 were more efficient in reducing gluconeogenesis from lactate/pyruvate than S 1475-1. Benfluorex inhibited gluconeogenesis at the level of pyruvate carboxylase (45% fall in acetyl-CoA concentration) and of glyceraldehyde-3-phosphate dehydrogenase (decrease in ATP/ADP and NAD^+/NADH ratios). Accordingly, neither benfluorex nor S 422-1 inhibited gluconeogenesis from dihydroxyacetone, but both stimulated gluconeogenesis from glycerol. In hepatocytes cultured in the presence of benfluorex or S 422-1 (10 or 100 μmol/l), the expression of genes encoding enzymes of fatty acid oxidation (carnitine palmitoyltransferase [CPT] I), ketogenesis (hydroxymethylglutaryl-CoA synthase), and gluconeogenesis (glucose-6-phosphatase, PEPCK) was decreased, whereas mRNAs encoding glucokinase and pyruvate kinase were increased. By contrast, Glut-2, acyl-CoA synthetase, and CPT II gene expression was not affected by benfluorex or S 422-1. In conclusion, this work suggests that benfluorex mainly via S 422-1 reduces gluconeogenesis by affecting gene expression and metabolic status of hepatocytes. *Diabetes* 51: 2363–2368, 2002

Human type 2 diabetes or animal models are characterized by hyperglycemia as a result of increased hepatic glucose production and peripheral insulin resistance (1). Type 2 diabetes is also associated with a profound alteration in lipid metabolism, such as increased levels of plasma very-low-density lipoprotein triglycerides (VLDL-TG) and free fatty acids, whose oxidation is increased in the liver and skeletal muscles (1). There is now increasing evidence that these disturbances in fat metabolism exacerbate the abnormalities in glucose metabolism in the liver and skeletal muscles (2,3). Indeed, accelerated fatty acid oxidation stimulates hepatic gluconeogenesis by providing acetyl-CoA, ATP, and reducing equivalents (NADH, $FADH_2$) (4) and reduces glucose utilization in peripheral tissues secondarily to an inhibition of pyruvate dehydrogenase activity (5). Therefore, a reduction in overall fatty acid oxidation should be expected to improve both hepatic glucose production and peripheral glucose oxidation. However, treatment with antilipolytic agents (e.g., acipimox, BRL 49658) has led to controversial data. For instance, overnight treatment of patients with type 2 diabetes with acipimox markedly improved glucose metabolism (6,7), whereas long-term treatment (1 month) was without effect on hepatic glucose production (8). Such discrepancies could be because during long-term treatment intrahepatic lipolysis would not be inhibited by acipimox. By contrast, 6-day treatment of patients with type 2 diabetes with etomoxir, an inhibitor of carnitine palmitoyltransferase I (CPT I) (the key regulatory enzyme of mitochondrial fatty acid oxidation), markedly decreased hepatic glucose production (9). However, such irreversible inhibitors of fatty acid oxidation had undesirable effects, such as cardiac hypertrophy and rise in plasma VLDL-TG and free fatty acids (10). Recently, troglitazone was shown to bypass such a disadvantage because it inhibited long-chain acyl-CoA synthetase (11) and thus induced a reduction in both hepatic fatty acid oxidation and triglyceride synthesis (11). It is interesting that benfluorex (1-(3-trifluoromethylphenyl)-2[*N*(2-benzyloxyethyl)amino]propane) was shown to have antihyperglycemic and hypolipidemic effects in diabetic animal models and in humans (12). Antihyperglycemic effects were mainly due to a decrease in hepatic gluconeogenesis and an increase in glucose utilization in both the liver (glycogenogenesis) and in oxidative skeletal muscles (12). The lipid-lowering effects of benfluorex were due to inhibition of hepatic lipogenesis and triglyceride and sterol synthesis (12). However, the causal relationship between these two effects of benfluorex has never been investigated.

The aim of the present study was to investigate the effect of benfluorex and two of its hepatic metabolites, S 422-1 and S 1475-1, on *1*) the interrelationships between fatty acid oxidation and gluconeogenesis in incubated

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ACS, acyl-CoA synthetase; B/A, β-hydroxybutyrate/acetoacetate; CPT, carnitine palmitoyltransferase; DHA, dihydroxyacetone; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GK, glucokinase; Glc-6-Pase, glucose-6-phosphatase; L-PK, liver-type pyruvate kinase; mtHMG-CoA synthase, mitochondrial hydroxymethylglutaryl-CoA synthase; VLDL-TG, very-low-density lipoprotein triglycerides.

TABLE 1
Effects of benfluorex and its metabolites on oleate metabolism in isolated hepatocytes from 24-h fasted rats

Additions	[1-^{14}C]Oleate converted (nmol/h per 10^6 hepatocytes)		Ketone body production (nmol/h per 10^6 hepatocytes)		
	CO_2	ASP	Acetoacetate	β-Hydroxybutyrate	B/A ratio
None	7.0 ± 0.5	42.3 ± 3.6	120 ± 11	83 ± 15	0.68 ± 0.11
Benfluorex					
0.1 mmol/l	9.0 ± 0.4	28.7 ± 2.2*	102 ± 12	25 ± 5*	0.26 ± 0.4*
1 mmol/l	13.8 ± 3.6*	22.8 ± 3.5†	74 ± 24	7 ± 5†	0.09 ± 0.02†
S 422-1					
0.1 mmol/l	7.3 ± 0.3	34.2 ± 2.6	121 ± 13	40 ± 7*	0.35 ± 0.06
1 mmol/l	11.1 ± 1.5*	28.6 ± 2.8*	116 ± 16	27 ± 15†	0.23 ± 0.07*
S 1475-1					
0.1 mmol/l	7.0 ± 0.4	37.4 ± 3.5	116 ± 13	56 ± 12	0.50 ± 0.11
1 mmol/l	8.7 ± 1.4	43.2 ± 3.5	123 ± 22	85 ± 15	0.69 ± 0.09
Metformin	4.4 ± 0.7	46.2 ± 6.3	31 ± 8†	171 ± 13†	5.6 ± 0.4†

Data are means ± SE of five to seven different experiments performed in duplicate. Hepatocytes were incubated for 1 h in the presence of [1-^{14}C]oleate (0.3 mmol/l) bound to 2% fat-free albumin and in the absence or in the presence of benfluorex or its metabolites at the indicated concentrations. Metformin (50 mmol/l) was used as a reference product. ASP, acid-soluble product (Krebs cycle intermediates + ketone bodies). *$P < 0.05$ and †$P < 0.01$ when compared with hepatocytes incubated without drugs.

hepatocytes and *2*) the expression of genes encoding specific proteins involved in these metabolic pathways in cultured hepatocytes. These experiments were carried out in hepatocytes isolated from 24-h fasted adult rats that exhibit the characteristics encountered in type 2 diabetes, such as an active fatty acid oxidation and an increased gluconeogenic rate.

RESEARCH DESIGN AND METHODS

Animals. Male Wistar rats (8–12 months old) that weighed 200–300 g were housed in individual plastic cages and were fed ad libitum a standard laboratory food (68% carbohydrate, 11% fat, and 21% protein in terms of energy). They were fasted for 24 h before hepatocyte isolation.

Isolation and incubation or culture of hepatocytes. Hepatocytes were isolated by in situ perfusion of the liver with 0.025% collagenase, as described previously (13). Hepatocytes (1–2 × 10^6 cells/ml) were incubated at 37°C in 2 ml of oxygenated (O_2:CO_2; 95:5) Krebs-Henseleit bicarbonate buffer (pH 7.4) for 1 h in a gyratory shaking water bath. Benfluorex (Mediator) and its metabolites S 422-1 and S 1475-1 were dissolved in DMSO and added (10 μl) to the incubation medium at a final concentration of 0.1 or 1 mmol/l. Metformin was used at a final concentration of 50 mmol/l as a reference antidiabetic compound. For the study of gene expression, isolated hepatocytes were plated in 75-cm^2 Petri dishes (3–5 × 10^6 cells/dish) and cultured in an M199 glucose-free medium as previously described (14). Benfluorex and its metabolites were used at a final concentration of 0.01 and 0.1 mmol/l.

Measurement of fatty acid oxidation. Fatty acid oxidation was studied using [1-^{14}C]oleate (0.3 mmol/l; 0.5 μCi/μmol) plus carnitine (0.5 mmol/l) and [1-^{14}C]octanoate (0.3 mmol/l; 0.1 μCi/μmol). Both fatty acids were bound to 2% (wt/vol) defatted albumin. Incubations were terminated by adding 0.2 ml $HClO_4$ (40% vol/vol). The production of $^{14}CO_2$ and labeled acid-soluble products was determined as previously described for [^{14}C]oleate (15) and [^{14}C]octanoate (16).

Glucose production rates and gluconeogenic intermediate concentrations. The rates of gluconeogenesis were determined after a 1-h incubation period in the absence (endogenous) or in the presence of lactate/pyruvate (10/1 mmol/l), dihydroxyacetone (10 mmol/l), or glycerol (10 mmol/l).

Gluconeogenic intermediate concentrations were measured in hepatocytes incubated for 1 h in the presence of alanine (10 mmol/l), in the absence of benfluorex (control), or in the presence of 1 mmol/l benfluorex. The incubations were terminated by adding 0.2 ml $HClO_4$ (40% vol/vol).

Isolation of mitochondria and measurement of long-chain acyl-CoA synthetase and carnitine palmitoyltransferase I activities. Mitochondria were isolated from 1-h incubated hepatocytes using the differential centrifugation technique according to Herbin et al. (17). The final mitochondrial pellet was resuspended at a protein concentration of 15 mg/ml. Protein concentrations were determined by the method of Lowry et al. (18). Mitochondrial CPT I activity was assayed in the presence of palmitoyl-CoA (80 μmol/l) and L-[methyl-^{3}H]carnitine (1 mmol/l; 1.6 Ci/mol) as described previously (17). The V_{max} of acyl-CoA synthetase was measured in the presence of [^{14}C]oleate (100 μmol/l) as described previously (11).

Metabolite analysis. Ketones, gluconeogenic intermediates, ATP, ADP, and acetyl-CoA concentrations were measured in the neutralized perchloric filtrates by enzymatic methods as described previously (19). Oxaloacetate was calculated according to the following formula:

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

where k_{MDH} and k_{LDH} represent, respectively, the equilibrium constants of malate dehydrogenase (2.78×10^{-5}) and lactate dehydrogenase (1.1×10^{-4}).

Extraction and Northern blot analysis of total RNA. Total RNA from frozen tissues and from hepatocytes of two Petri dishes were extracted with guanidium thiocyanate followed by a purification through a CsCl cushion gradient according to Chirgwin et al. (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 as previously described (21). Hybridization of the blots with an excess of [γ-^{32}P]ATP-labeled 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 were described in detail in Fulgencio et al. (14). They were radiolabeled using the multiprime DNA labeling system (Amersham). Quantifications were performed by scanning densitometry of the autoradiographs.

Statistical analysis. Results are expressed as means ± SE. Statistical analysis was performed using the rank-order test (23).

RESULTS

Effect of benfluorex and its metabolites on fatty acid oxidation rates. Benfluorex decreased, in a concentration-dependent manner, the synthesis of acid-soluble products and ketone bodies from oleate (Table 1), whereas the production of $^{14}CO_2$ into citric acid cycle was markedly increased by benfluorex, probably as the result of a fall in the β-hydroxybutyrate/acetoacetate (B/A) ratio (Table 1). This more oxidized redox state in mitochondria should favor the NAD^+-linked isocitrate dehydrogenase/2-oxoglutarate dehydrogenase complex. Conversely, metformin, which had no effect on oleate oxidation and total ketone body synthesis (Table 1), induced a huge increase in the B/A ratio. This high reduced mitochondrial redox state was associated with a decrease in $^{14}CO_2$ production from

TABLE 2
Effects of benfluorex and S 422-1 on octanoate metabolism and long-chain ACS and CPT I activities

	Control	Benfluorex (1 mmol/l)	S 422-1 (1 mmol/l)
[1-^{14}C]Octanoate converted (nmol/h per 10^6 hepatocytes)			
CO_2	7.2 ± 1.0	11.6 ± 0.9	12.7 ± 1.1
ASP	166 ± 6	105 ± 7†	97 ± 9†
β-Hydroxybutyrate	124 ± 16	42 ± 9†	55 ± 4†
Acetoacetate	128 ± 3	77 ± 9*	93 ± 5*
B/A ratio	0.96 ± 0.13	0.53 ± 0.05*	0.59 ± 0.04*
ACS (μU/mg mitochondrial protein)	46 ± 10	49 ± 8	34 ± 9
CPT (nmol · min^{-1} · mg^{-1} mitochondrial protein)	4.1 ± 0.2	4.7 ± 0.2	3.9 ± 0.5

Data are means ± SE of four different experiments performed in duplicate. Hepatocytes were incubated for 1 h in the presence of [1-^{14}C]octanoate (0.6 mmol/l) bound to 2% fat-free albumin and in the absence or in the presence of benfluorex and S 422-1 at the indicated concentration. *$P < 0.05$ and †$P < 0.01$ when compared with hepatocytes incubated without drugs.

oleate (Table 1). The effects of benfluorex on fatty acid metabolism were entirely reproduced by its metabolite S 422-1, whereas S 1475-1 had no significant effect on any of the parameters studied (Table 1). Neither long-chain acyl-CoA synthetase (ACS) nor CPT I maximal activities were affected by benfluorex or S 422-1 (Table 2), suggesting that the inhibitory effect of these compounds resides inside the mitochondria. Indeed, the oxidation of octanoate, which is independent of long-chain ACS or CPT I activities, was also reduced by benfluorex and S 422-1 (Table 2).

Effect of benfluorex and its metabolites on hepatic gluconeogenesis. As shown in Fig. 1, benfluorex and S 422-1 inhibited in a dose-dependent manner the rates of gluconeogenesis from lactate/pyruvate (10/1 mmol/l). At the highest concentration, both benfluorex and S 422-1 were as efficient as metformin in reducing gluconeogenesis (Fig. 1). S 1475-1 significantly reduced gluconeogenesis only when used at a concentration of 1 mmol/l (Fig. 1). Because benfluorex decreased β-oxidation (Tables 1 and 2), we wondered whether this could explain in part the reduction of gluconeogenesis. The effect of benfluorex (1 mmol/l) on gluconeogenic intermediate concentrations was determined in the presence of oleate (0.3 mmol/l) and alanine (10 mmol/l). Benfluorex, which produced a 57 ± 4% inhibition of gluconeogenesis from alanine (70 ± 6 vs. 164 ± 16 nmol/h per 10^6 hepatocytes; $n = 6$, $P < 0.01$), induced two crossovers (Fig. 2). The first crossover between pyruvate and oxaloacetate/malate suggested an inhibition of pyruvate carboxylase as the result of a 43 ± 5% decrease in acetyl-CoA concentration in benfluorex-treated hepatocytes (0.67 ± 0.05 vs. 1.16 ± 0.11 nmol/10^6 hepatocytes; $n = 4$, $P < 0.05$). The second crossover between 3-phosphoglycerate and dihydroxyacetone phosphate (Fig. 2) suggested an inhibition of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and/or 3-phosphoglycerate kinase. As the cofactors involved in these two reactions were affected by benfluorex, it was difficult to differentiate between these two potential sites of regulation. Indeed, the ATP/ADP ratio was decreased by 60% by benfluorex (1.8 ± 0.3 vs. 4.8 ± 0.3; $n = 8$, $P < 0.01$). Similarly, the cytosolic NAD^+/NADH ratio was decreased in benfluorex-treated hepatocytes as reflected by the fall in the lactate/pyruvate ratio (2.0 ± 0.4 vs. 3.7 ± 0.3 for control; $n = 6$, $P < 0.05$).

If benfluorex and S 422-1 inhibited gluconeogenesis only by reducing β-oxidation, then it would be expected that gluconeogenesis from a substrate that enters the pathway downstream of the reaction catalyzed by GAPDH, such as dihydroxyacetone (DHA), would not be inhibited. Indeed, gluconeogenesis from DHA was not affected by benfluorex or its two metabolites (1 mmol/l) (control, 519 ± 20; benfluorex, 523 ± 42, S 422-1, 557 ± 19; S 1475-1, 513 ± 39 nmol/h per 10^6 hepatocytes; $n = 6$). Moreover, if the reduction in gluconeogenesis from lactate/pyruvate was mainly due to the more oxidized redox state induced by benfluorex and S 422-1, then the rates of gluconeogenesis should be enhanced by these compounds as the consequence of activation of GAPDH (24). Indeed, the rates of gluconeogenesis from glycerol were increased by 35 and 25%, respectively, by benfluorex and S 422-1 (1 mmol/l) (control, 100 ± 5; benfluorex, 135 ± 6; S 422-1, 126 ± 7 nmol/h per 10^6 hepatocytes; $n = 4$, $P < 0.05$).

Effect of benfluorex and S 422-1 on hepatic gene expression. Because at the doses used S 1475-1 had no effect on the expression of genes investigated in the present study, only the effects of benfluorex and S 422-1 are presented. Two classes of genes were studied: those coding for regulatory proteins of glucose metabolism and

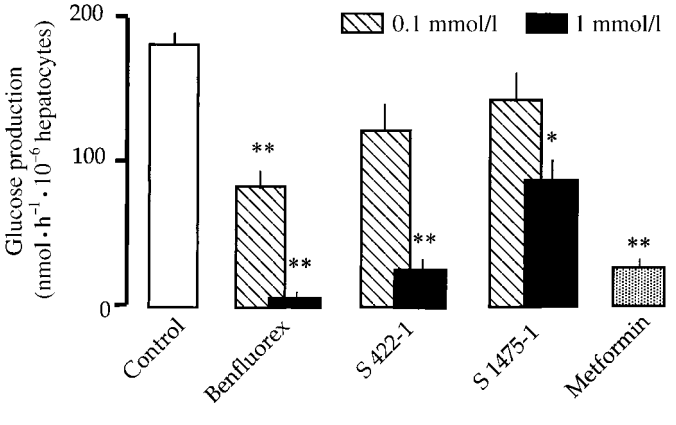

FIG. 1. Dose-dependent effect of benfluorex and its metabolites S 422-1 and S 1475-1 on hepatic glucose production from lactate/pyruvate (10/1 mmol/l). Hepatocytes were incubated for 1 h in the absence (control) or in the presence of each compound at the indicated concentration. Metformin (50 mmol/l) was used as reference compound. Endogenous glucose production (no substrate added for 1 h) was subtracted from each value. Results are means ± SE of five to seven different experiments. *$P < 0.05$ and **$P < 0.01$ when compared with control hepatocytes.

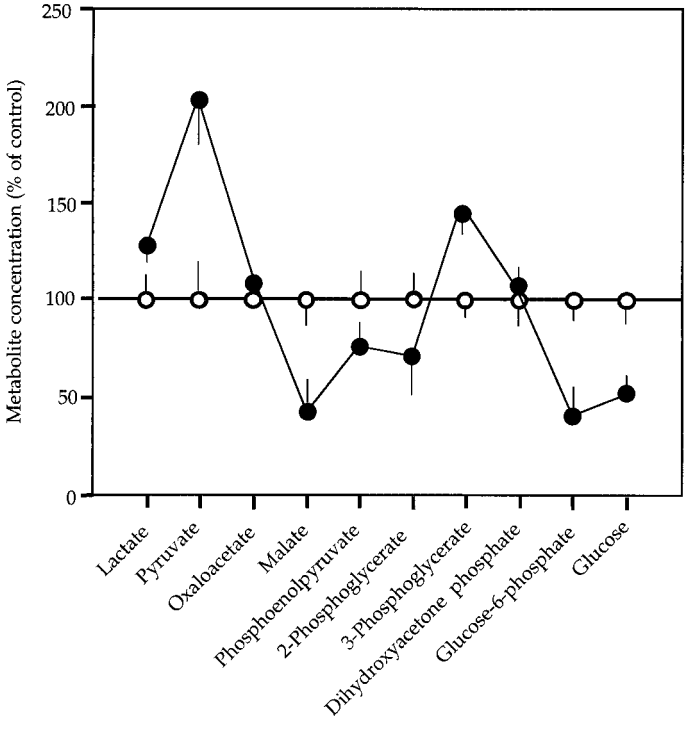

FIG. 2. Crossover plot showing the effect of benfluorex on the concentration of gluconeogenic intermediates in isolated hepatocytes incubated for 1 h in the presence of alanine (10 mmol/l). The metabolite concentration in 1 h benfluorex-treated hepatocytes (1 mmol/l; ●) was expressed as a percentage of that found in control hepatocytes (○). The concentration of each metabolite in control cells is given in nanomoles per 10^6 hepatocytes: lactate, 62 ± 8; pyruvate, 17 ± 3; calculated oxaloacetate, 0.3; malate, 3.1 ± 0.6; phosphoenolpyruvate, 2.3 ± 0.5; 2-phosphoglycerate, 0.6 ± 0.2; 3-phosphoglycerate, 3.5 ± 0.7; dihydroxyacetone phosphate, 3.8 ± 0.8; glucose-6-phosphate, 0.52 ± 0.13; glucose, 163 ± 18. Results are means ± SE of six different experiments performed in duplicate.

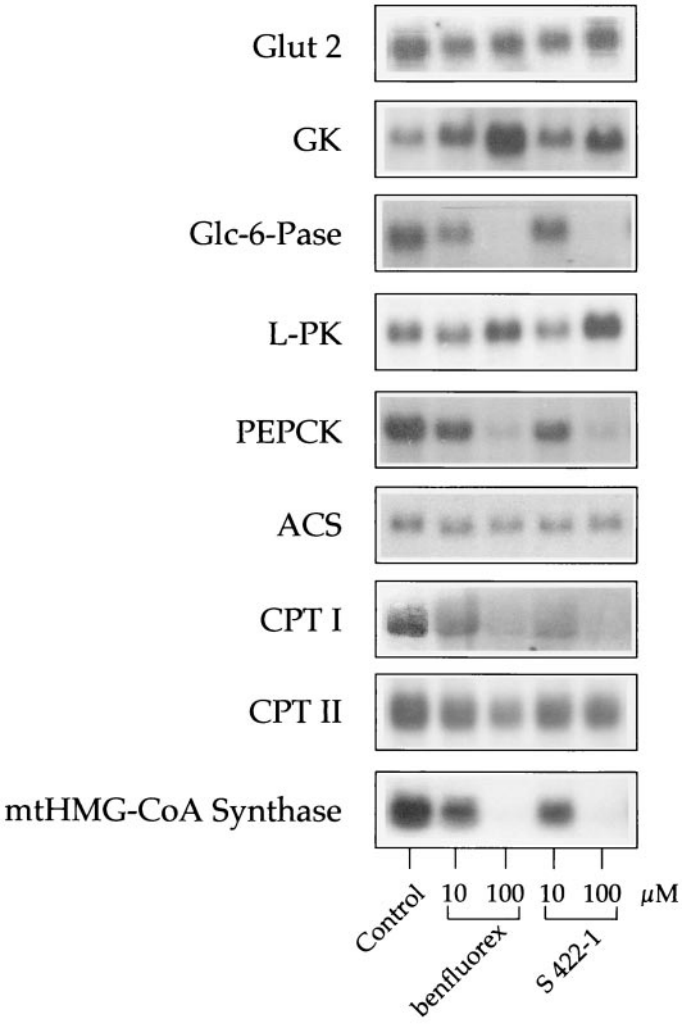

FIG. 3. Representative Northern blot of the effect of benfluorex and S 422-1 on mRNA level encoding glucose transporter Glut 2, GK, Glc-6-Pase, L-PK, PEPCK, long-chain ACS, CPT I, CPT II, and mtHMG-CoA synthase.

those encoding key enzymes of mitochondrial fatty acid metabolism.

Neither benfluorex nor S 422-1 affected the expression of the hepatic glucose transporter Glut-2, whatever the concentration used (Fig. 3, Table 3). By contrast, the two compounds had opposite effects on the expression of glycolytic and gluconeogenic enzymes. Both glucokinase (GK) and liver-type pyruvate kinase (L-PK) mRNA levels were increased by the highest concentration of benfluorex and S 422-1 (Table 3). Conversely, benfluorex and S 422-1 reduced in a concentration-dependent manner the expression of glucose-6-phosphatase (Glc-6-Pase) and PEPCK genes (Fig. 3, Table 3). These effects of benfluorex and S 422-1 were similar to those observed for metformin on the expression of glycolytic and gluconeogenic enzymes (Table 3). By contrast, there are marked differences between the effects of benfluorex and S 422-1 on genes encoding protein of fatty acid metabolism and the effect of metformin on these genes. Whereas benfluorex and S 422-1 markedly reduced, in a concentration-dependent manner, the expression of CPT I and mitochondrial hydroxymethylglutaryl-CoA synthase (mtHMG-CoA synthase), metformin had no significant effect on these genes (Fig. 3, Table 3). Benfluorex, S 422-1, and metformin had no effect on the expression of ACS and CPT II genes (Fig. 3, Table 3).

DISCUSSION

In the liver, benfluorex is rapidly split into metabolites, among which S 422-1 (1-(3-trifluoromethylphenyl)-2-[*N*-(2-hydroxyethyl)amino] propane) represents the main bioactive compound (12).

As already mentioned in the introduction of this article, the beneficial effects of fatty acid oxidation inhibitors are still questioned because most of these compounds induce the accumulation of triglycerides both in the liver (4) and in skeletal muscle cells (25). Indeed, there is growing evidence that the degree of insulin resistance is tightly correlated with the excessive accumulation of fat in the liver and skeletal muscles. Benfluorex should bypass this disadvantage because, despite its inhibitory effect on mitochondrial β-oxidation, it reduces serum lipid concentration both in animal models (26) and in humans (27) as a result of an inhibition of lipogenesis and triglyceride synthesis (28). Our work also demonstrates that the reduction in plasma ketone body concentration in humans (27) and ketone body production in rat hepatocytes (28) observed after benfluorex treatment results from an inhibition of mitochondrial β-oxidation. Even though benfluorex does not affect CPT I activity, it seems unlikely that it

TABLE 3
Dose-dependent effect of benfluorex and its metabolite on mRNA levels of genes encoding regulatory proteins of glucose and fatty acid metabolism in cultured hepatocytes from 24-h fasted rats

	% of control value				
	Benfluorex		S 422-1		Metformin
	10 μmol/l (n = 6)	100 μmol/l (n = 6)	10 μmol/l (n = 6)	100 μmol/l (n = 6)	500 μmol/l (n = 6)
Hepatic genes					
Glut-2	82 ± 9	88 ± 14	72 ± 16	88 ± 5	110 ± 19
Glycolysis/gluconeogenesis					
GK	101 ± 16	175 ± 31*	108 ± 22	168 ± 16*	166 ± 30*
Glc-6-Pase	86 ± 12	51 ± 17*	72 ± 11*	32 ± 7†	70 ± 8*
L-PK	80 ± 13	135 ± 12*	88 ± 10	145 ± 4†	230 ± 30*
PEPCK	74 ± 6*	24 ± 3†	51 ± 4†	12 ± 2†	53 ± 9†
Fatty acid oxidation/ketogenesis					
ACS	90 ± 15	121 ± 18	103 ± 20	120 ± 10	120 ± 26
CPT I	74 ± 8*	34 ± 8†	59 ± 6†	20 ± 4†	80 ± 10
CPT II	98 ± 11	111 ± 13	100 ± 8	114 ± 10	108 ± 10
mtHMG-CoA synthase	84 ± 13	12 ± 4†	65 ± 9*	7 ± 2†	76 ± 16

Results are means ± SE and are expressed as percentage of the mRNA levels found in hepatocytes cultured in control conditions. Total RNA were extracted from 24-h fasted adult rat hepatocytes cultured in the absence or in the presence of benfluorex or S 422-1 at the indicated concentrations. Metformin was used as a reference product.

reduces β-oxidation through a malonyl-CoA–dependent mechanism because it decreases hepatic lipogenesis (28) and the oxidation of octanoate, which is independent of the CPT I activity.

This work also provided evidence that the mechanisms by which benfluorex reduces hepatic gluconeogenesis are markedly different from those of metformin, the main antidiabetic compound used in the world. Indeed, reduction of gluconeogenesis by metformin is independent of the rate of β-oxidation (present work, 14), whereas several lines of evidence suggest that inhibition of gluconeogenesis by benfluorex is, at least in part, due to a decrease in mitochondrial β-oxidation. First, benfluorex decreases acetyl-CoA concentration, which in turn would reduce pyruvate carboxylase activity and release its inhibitory effect on pyruvate dehydrogenase (29). Second, benfluorex decreases both the ATP-to-ADP and the NAD^+-to-NADH ratios, leading to a reduced gluconeogenic flux at the level of 3-phosphoglycerate kinase and GAPDH. Changes in cellular redox state represent probably the main mechanism by which benfluorex reduces glucose production in hepatocytes because *1*) gluconeogenesis from DHA, a substrate that enters this pathway upstream of GAPDH, is not inhibited by benfluorex and S 422-1 and *2*) gluconeogenesis from glycerol is increased by benfluorex and S 422-1 as the result of an activation of GAPDH (24) as a result of the more oxidized redox state in benfluorex-treated hepatocytes. It must be stressed that a decrease in mitochondrial pyruvate carrier has also been shown as a putative mechanism involved in the reduction of gluconeogenesis during acute (short-term) exposure to benfluorex (28). During chronic treatment of adult rat with benfluorex, the fall in PEPCK activity was shown to be responsible for the decrease in hepatic gluconeogenesis (30). However, the question arising from these in vivo experiments was whether the decrease in enzyme activity resulted from a direct effect of benfluorex on gene expression or from an indirect effect via a benfluorex-induced increase in insulin sensitivity (12). The decrease in PEPCK gene expression in hepatocytes cultured in the absence of insulin could represent a nonexclusive explanation for the fall in PEPCK activity after chronic (long-term) benfluorex treatment. Indeed, the present work provides evidence that both benfluorex and S 422-1 affect directly the expression of genes encoding regulatory protein of fatty acid oxidation, gluconeogenesis, and glycolysis. Such effect of benfluorex or S 422-1 on gene expression has not been reported previously either in vivo or in vitro except for the glucose transporter Glut-4, whose expression is regulated by benfluorex at a translational/posttranslational level in white skeletal muscle (31). Unfortunately, the delay between changes in mRNA level and changes in protein did not allow metabolic studies because after 48 h of culture, hepatocytes lost most of their differentiated functions, especially the capacity to perform an efficient fatty acid oxidation and ketogenesis (data not shown). However, if we assume that modifications in gene expression are associated with corresponding changes in enzyme activities, then benfluorex through S 422-1 should have antihyperglycemic effects via a combined stimulation of glycolysis and inhibition of gluconeogenesis at the level of Glc-6-Pase/GK and of PEPCK/L-PK cycles. Although the molecular basis of these changes remains to be determined, the present work demonstrates that benfluorex or its metabolite controls gene expression in an insulin-independent pathway. It is interesting that similar observations were observed with troglitazone, another insulin sensitizer (32) that inhibits PEPCK gene expression in vitro by an insulin-independent, antioxidant-related mechanism (33). Despite similarities in the effects of troglitazone and benfluorex on cellular redox state in vitro (present work, 11), we cannot assume that the factors involved in the regulation of these genes could be the same. However, it is interesting to speculate that metabolic changes induced by acute benfluorex treatment (e.g., redox state, ATP status) could be involved in the regulation of gene expression, because these metabolic factors tightly control the level and/or the degree of phosphoryla-

tion of ubiquitous transcription factors (e.g., activator protein-1, Sp1, nuclear factor-κB) involved in the control of numerous genes (34). Another "economical" way to orchestrate complex transcriptional response to a given compound is to affect the expression of proteins that interact with transcription factors, such as coactivators. It was shown recently that PGC-1, a coactivator that interacts with many transcription factors (35), is a powerful regulator of hepatic gluconeogenesis during fasting or in diabetic animal models (36,37). Such putative mechanisms of action of benfluorex are currently under investigation.

In conclusion, this work demonstrates that the decrease in gluconeogenesis induced by benfluorex and S 422-1 is due to the reduction in cofactor availability (acetyl-CoA, NADH, and ATP) as the result of the inhibition of β-oxidation. Moreover, this study shows that benfluorex and S 422-1 control in vitro the expression of genes encoding regulatory proteins involved in fatty acid and glucose metabolism by an insulin-independent mechanism.

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