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S-15261, a new anti-hyperglycemic agent, reduces hepatic glucose production through direct and insulin-sensitizing effects

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Abstract

S-15261 is a new oral anti-hyperglycemic agent that increases insulin sensitivity in various insulin-resistant animal models. The aim of this study was to determine the short- and long-term effects of S-15261 and its metabolites (S–15511 and Y-415) on fatty acid and glucose metabolism in hepatocytes isolated from 24-h starved rats. During short-term exposure (1 h) neither S-15261 nor its metabolites affected fatty acid oxidation whatever the concentration used. By contrast, S-15261 and its two metabolites reduced the rates of glucose production from lactate/pyruvate and dihydroxyacetone. Using crossover plot analysis, it was shown that Y-415 reduced hepatic gluconeogenesis upstream the formation of dihydroxyacetone phosphate. After 48 h in culture, S-15261 and its two metabolites reduced the rates of glucose production from lactate/pyruvate secondarily to a decrease in PEPCK and Glc-6-Pase mRNA levels. A part of these effects on gene expression could be due to a drug-induced reduction in PGC-1 gene expression. When hepatocytes were cultured in the presence of a submaximal concentration of insulin (10^{-9} M), S-15261, through its metabolite S-15511, enhanced insulin sensitivity both on gene expression (PEPCK, Glc-6-Pase, PGC-1) and on gluconeogenesis. Furthermore, S-15261 and S-15511 induced the expression of GK and FAS genes as the result of an increased in SREBP-1c mRNA levels. Finally, S-15511 enhanced the stimulatory effect of insulin on GK mRNA level through an additional increase in SREBP-1c gene expression.

In conclusion, this work reveals that S-15261 via its metabolites reduces hepatic glucose production through direct and insulin-sensitizing effects on genes encoding regulatory proteins of hepatic glucose metabolism.

Keywords: Anti-diabetic agent; Gluconeogenesis; Fatty acid oxidation; Gluconeogenic; Glycolytic and lipogenic gene expression

1. Introduction

Insulin resistance, and hyperinsulinemia are characteristic features of type 2 diabetes, obesity and also of essential hypertension, hyperlipidemia, atherosclerosis and ageing (Reviewed in: [1,2]). They occur in peripheral tissues, in which insulin-mediated glucose uptake is reduced and in the liver, where glucose production is inappropriately high for the prevailing plasma glucose and insulin levels (Reviewed in: [3]). Treatment of insulin-resistant patients focused on weight reduction either by diet or exercice are often ineffective (Reviewed in: [4]). Therefore, the absence of fully effective treatments for the insulin resistance syndrome has prompted intensive efforts to develop pharmacological agents that would increase insulin sensitivity and reduce the associated hyperinsulinemia. Drugs such as thiazolidinediones or benfluorex have both lipid-lowering and insulin-sensitizing effects [5–7] whereas metformin reduces hepatic gluconeogenesis. Thus the resulting decrease in hyperglycemia produced by these drugs could improve peripheral insulin resistance (Reviewed in: [8]).

Recently, a novel oral agent, S-15261, was reported to increase insulin sensitivity in various insulin-resistant animal models [9–11]. This compound is the L-isomer of 3-[2-[2-[4-[2-[α-fluorenyl) acetyl] aminoethyl) benzoyloxy] ethylamino]1-methoexyethyl trifluoro methyl-benzene (Fig. 1) and contains an ester linkage that is cleaved by plasma esterases. Cleavage yields the fragments Y-415 [4-[2-[2-(9H-fluoren-9-yl)acetamido]ethyl) benzoic acid] and S-15511 [(−)-5-methoxy-5-[3-trifluoromethyl)phenyl]-3-

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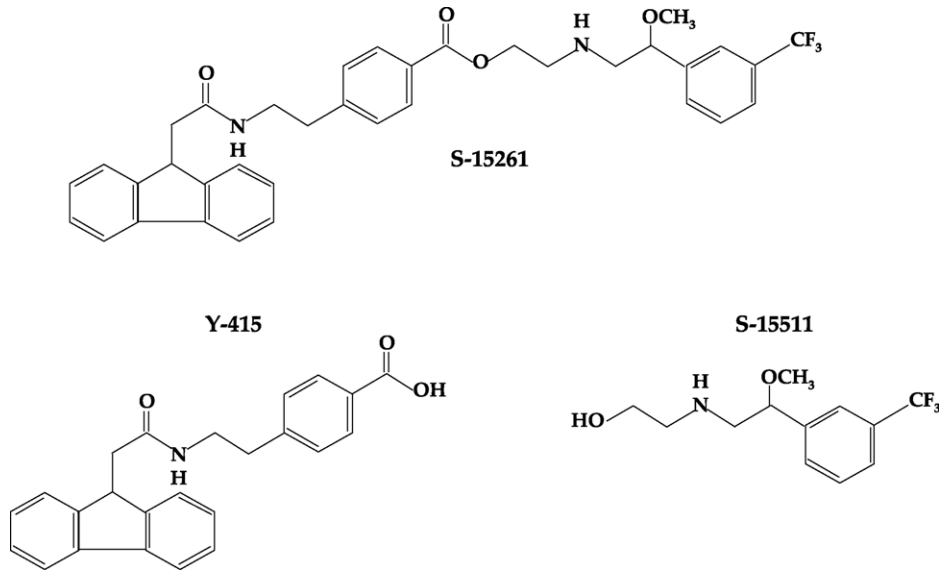

Fig. 1. The chemical structures of S-15261, S-15511 and Y-415 shown as the free bases.

azapentanol] (the chemical structure of S-15261 and its metabolite is given in Fig. 1). The two major metabolites of S-15261 are responsible for the biological activity of the parent drug; Y-415 causes a significant increase in insulin secretion from isolated mouse pancreatic islets [10,12] while S-15511 seems to be responsible for the insulin enhancing effects of S-15261 [11]. Whether directly or as the result of enhanced insulin sensitivity, it has been shown that chronic treatment of insulin-resistant animals with S-15261 normalized plasma glucose levels and reduced the circulating concentrations of triglycerides and fatty acids [10,11,13,14]. Taken together these data, suggest that S-15261 could be a promising agent for the treatment of type 2 diabetes and its associated cardiovascular complications. Although the mechanism of action of S-15261 is not fully understood, a recent study has shown that the reduction of hepatic glucose production and the lipid lowering effect of this compound may be due to changes in the expression of specific genes [11].

However, in vivo studies did not allow to discriminate between direct effect of the drug and/or indirect effects due to improved insulin secretion and sensitivity. Thus the aim of the present study was to investigate the in vitro effects of S-15261 and its two major metabolites on hepatic glucose and fatty acid metabolism. The short-term effects of these compounds on hepatic gluconeogenesis, fatty acid oxidation and the interrelations between these two pathways were studied in incubated hepatocytes whereas effects on gene expression were studied in hepatocytes cultured in the absence or in the presence of submaximal concentration of insulin. In both studies hepatocytes were isolated from 24-h fasted rats that exhibit some characteristics of the diabetic syndrome, such an active fatty acid oxidation and increased gluconeogenic rates.

2. Material and methods

2.1. Animals

Male Wistar rats (6 week-old) weighting 200–250 g were housed in individual plastic cages at 24 °C with light from 07:00 to 19:00 h. They had free access to water and were fed ad-libitum a standard laboratory chow pellet (68% carbohydrate, 11% fat, 21% protein in term of energy). They were fasted for 24 h before hepatocyte isolation.

2.2. Isolation, incubation and culture of hepatocytes

Hepatocytes were isolated by in situ perfusion of liver with 0.013% collagenase, as previously described [15]. Hepatocytes (1–2 $\times$ 10^6 cells/ml) were incubated at 37 °C in 2 ml of oxygenated (O_2/CO_2; 95:5) Krebs–Henseileit bicarbonate buffer (pH 7.4) for 1 h in a giratory shaking water bath. Each experiment was performed in duplicate. S-15261 and its metabolites S-15511 and Y-415 were provided by the Institut de Recherches Internationales Servier. They were dissolved in dimethyl sulfoxide (DMSO) and added (10 μl) to the incubation medium at a final concentration of 0.01, 0.1 or 1 mmol/l. The addition of 10 μl of DMSO into control flasks did not affect fatty acid or glucose metabolism (data not shown). For the study of gene expression in culture, hepatocytes were plated in 60 cm^2 petri dishes (2.5 $\times$ 10^6 cells/dish) in a M199 glucose-free medium containing penicillin (10 UI/ml), streptomycin (100 μg/ml). During cell attachement (4 h) a substitute of fetal calf serum (Ultroser G, 2%; Biosepra, France) was present. Duplicate dishes were used for all experimental conditions. The cultures were maintained for 24 or 48 h at 37 °C in an incubator equilibrated with

O_2/CO_2 (95:5). S-15261 and its metabolites were used at a final concentration of 0.01 and 0.1 mmol/l.

2.3. Measurement of fatty acid oxidation

Long-chain fatty acid metabolism was studied using [1-^{14}C] oleate (0.3 mmol/l; 56 μCi/μmol) plus carnitine (1 mmol/l). Oleate was bound to (2%, w/v) defatted albumin. Incubations were ended by adding 0.2 ml of $HClO_4$ (40%, v/v). The production of $^{14}CO_2$ and labelled acid-soluble products were determined as described in [16].

2.4. 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 either a mixture of lactate/pyruvate (10/1 mmol/l) or dihydroxyacetone (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 Y-415 (control) or in the presence of Y 415 (0.1 mmol/l). The incubations were ended by adding 0.2 ml of $HClO_4$ (40%, v/v).

Glucose production rates from lactate/pyruvate (10/1 mmol/l) were also determined in hepatocytes cultured for 48 h with each compound in the absence or in the presence of 10^{-9} M of insulin.

2.5. Metabolite analysis

Ketones and gluconeogenic intermediates concentrations were measured in the neutralized perchloric filtrates by enzymatic methods as described previously [17]. 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, respectively the equilibrium constants of malate dehydrogenase (2.78×10^{-5}) and lactate dehydrogenase (1.1×10^{-4}).

2.6. Extraction and analysis of mRNA level by real-time quantitative PCR

Total cellular RNAs from hepatocytes of two Petri dishes were extracted by using the guanidinium thiocyanate method [18]. Total RNA (500 ng) were reverse transcribed for 1 h at 42 °C in a 20 μl final volume reaction containing 50 mM Tris–HCl, 75 mM KCl, 3 mM MgCl2, 10 mM DTT, 250 ng random hexamers (Promega), 250 ng oligo(dT) (Promega), 0.75 mM of each dNTPs and 100 units of superscript II reverse transcriptase (Invitrogen). Real time quantitative PCR analysis was performed starting with 2.0 ng of reverse transcribed total RNA, in a final volume of 10 μl PCR reaction, with 0.5 μM of each primer (Invitrogen), 3 mM $MgCl_2$, using 1x light cyclerDNA Master SYBR Green I mix in a light cycler instrument (Roche Molecular Biochemicals). Samples were incubated in the light cycler apparatus for an initial denaturation at 95 °C for 10 min, followed by 40 cycles. Each cycle consisted of 95 °C for 15 s, 58 °C for 7 s, and 72 °C for 15 s. The specific primers used are described in Table 1. SYBR Green I fluorescence emission was determined after each cycle. The relative amounts of the different mRNAs were quantified by using the second derivative maximun method of the light-cycler-software. Cyclophilin was used as an invariant control and the relative quantification for a given gene was corrected to the cyclophilin mRNA values. Amplification of specific transcripts was confirmed by melting curves profiles generated at the end of each run. PCR specificity and product length were further checked by agarose gel electrophoresis and ethidium bromide staining.

2.7. Statistical analysis

Results are expressed as mean ± S.E.M. Statistical analysis was performed using the rank-order test [19].

Table 1
RT-PCR primers

Gene		Sequence	Length
Cyclophilin	Sense	5′ ATGGCACTGGTGGCAAGTCC 3′	
	Anti-sense	5′ TTGCCATTCCTGGACCCAAA 3′	241 pb
Glucokinase	Sense	5′ CAACATCGTAGGACTTCTCCG 3′	
	Anti-sense	5′ GGCGGTCTTCATAGTAGCAG 3′	119 pb
Glucose-6-phosphatase	Sense	5′ TCTTGTGGTTGGGATACTGG 3′	
	Anti-sense	5′ GCAATGCCTGACAAGACTC 3′	109 pb
PEPCK	Sense	5′ TGGCTACGTCCCTAAGGAA 3′	
	Anti-sense	5′GGTCCTCCAGATACTTGTCGA 3′	131 pb
FAS	Sense	5′ ACCTGTCCCAGGTGTGTGAT 3′	
	Anti-sense	5′ GCTGTGGATGATGTTGATGA 3′	111 pb
SREBP-1c	Sense	5′ TTGAAGACATGCTTCAGCTC 3′	
	Anti-sense	5′ GCCTGTGTCTCCTGTCTAC 3′	95 pb
PPAR gamma coactivateur 1	Sense	5′ TAAACTGAGCTACCCTTGGG 3′	
	Anti-sense	5′ CTCGACACGGAGAGTTAAAGGAA 3′	89 pb

3. Results

3.1. Effects of S-15261 and its two metabolites (S-15511 and Y-415) on glucose production rates

In the absence of the exogenous lactate/pyruvate mixture, the rate of glucose production was very low (12 ± 2 nmol/h/10^6 hepatocytes, $n = 11$) due to the exhausted glycogen stores in fasted rat hepatocytes. Thus the effects of various compounds will be ascribable to hepatic gluconeogenesis. As shown on Fig. 2, each compound reduced in a dose-dependent manner the rate of glucose production from lactate/pyruvate. Whatever the concentration used, Y-415 appeared to have the greatest effect on hepatic gluconeogenesis (Fig. 2).

3.2. Effects of S-15261 and its metabolites S-15511 and Y-415 on oleate oxidation

As shown on Table 2 none of these compounds significantly affected the rate of oleate oxidation (CO_2 plus acid soluble products) whatever the concentration used 0.1 or 1 mmol/l. This absence of effect of S-15261 and its metabolites on fatty acid oxidation was not due to a low sensitivity of incubated hepatocytes since in similar set of experiments troglitazone or benfluorex markedly inhibited fatty acid oxidation [20,21].

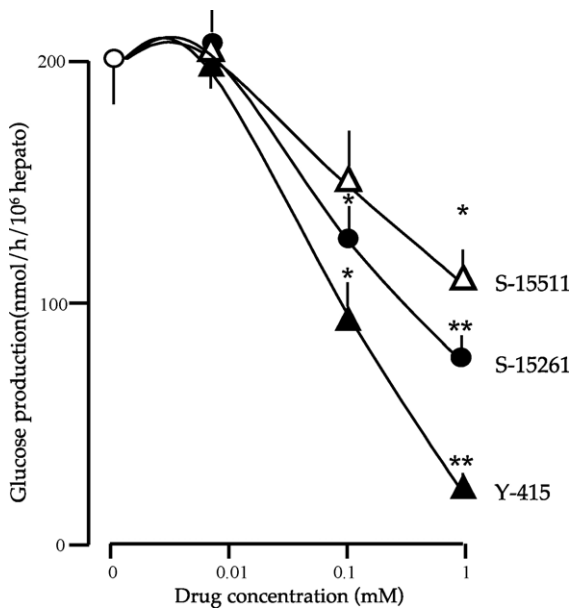

Fig. 2. Dose-response curve of the effect of S-15261 and its metabolites S-15511 and Y-415 on hepatic glucose production from lactate/pyruvate (10/1 mmol/l). Hepatocytes were incubated for 1 h in the absence (○) or in the presence of S-15261 (●), S-15511 (△) or Y-415 (▲) at the indicated concentration. Endogenous glucose production (no substrate added for 1 h) was subtracted from each value. Results are mean ± S.E.M. of five to seven different experiments. $^{*}P < 0.05$ and $^{**}P < 0.01$ when compared to control hepatocytes.

3.3. Effect of Y-415 on the concentration of gluconeogenic intermediates

Data from Fig. 2 demonstrated that during short tem exposure, Y-415 was the most efficient metabolite of S-15261 in reducing hepatic glucose production. To localize the putative(s) step(s) affected by Y-415 a cross-over plot analysis with alanine as gluconeogenic substrate was used. Results presented in Fig. 3 showed a clear crossover between dihydroxyacetone phosphate and glucose-6-phosphate. These results are consistent with the fact that gluconeogenic steps upstream dihydroxyacetone phosphate synthesis are not controlled by cellular redox state [17]. Indeed, Y-415 failed to affect the cytosolic $NADH/NAD^+$ ratio estimated from the ratio of concentration of lactate/pyruvate (3.5 ± 0.4 for control versus 4.6 ± 0.5 for Y-415; $n = 6$) as expected from the absence of effect on oleate oxidation rates (Table 2). Moreover, the rate of glucose production measured from dihydroxyacetone are markedly reduced by Y-415 (293 ± 38 or 174 ± 24 versus 439 ± 37 nmol/h/10^6 hepatocytes, respectively for Y-415 0.1 or 1 mM versus control $n = 6$; $P < 0.01$). Interestingly, S-15261 inhibited glucose production from dihydroxyacetone (322 ± 30 and 258 ± 30, respectively at 0.1 or 1 mM versus 439 ± 37 nmol/h/10^6 hepatocytes for control $n = 6$; $P < 0.01$) whereas S-15511 had modest effect when used at the higher concentration (1 mM; 340 ± 44 nmol/h/10^6 hepatocytes $n = 6$; $P < 0.05$). These results suggest that during short-term exposure, the reduction of gluconeogenesis by S-15261 is mainly due to Y-415.

3.4. Effect of S-15261 and its metabolites S-15511 and Y-415 on gluconeogenesis in cultured hepatocytes

Increasing the time of exposure from 1 h (incubation) to 48 h (culture) to S-15261 or its metabolites led to similar reduction in the rates of hepatic glucose production as previously reported in incubation experiments (ranged from 25 to 55%, Table 3) This decrease in hepatic glucose production occurred at concentration of each compound 10-fold lower than in incubated hepatocytes (Fig. 2). Moreover, when Y-415 was the most efficient metabolite in incubated hepatocytes, data from Table 3 showed that during long-term exposure, S-15511 was as efficient as Y-415 in reducing hepatic gluconeogenesis. From in vivo experiments, it was shown that S-15261 increased insulin sensitivity in various insulin-resistant animal models [9–11]. In order to determine which metabolite of S-15261 was responsible for the insulin sensitizing effect observed in vivo, the effects of S-15261 and its two metabolites were analyzed in hepatocytes cultured in the presence of a concentration of insulin inducing a half-maximal reduction in hepatic gluconeogenesis. As shown on Table 3, when hepatocytes were cultured in the presence of 10^{-9} M insulin, the addition of S-15261 or S-15511 induced a

Table 2
Effects of S-15261 and its metabolites on oleate metabolism in isolated hepatocytes from 24-h starved rats

	[1-^{14}C] Oleate converted (nmol/h/10^6 hepatocytes)		Ketone body production (nmol/h/10^6 hepatocytes)		
Additions	CO_2	ASP	Acetoacetate	β-Hydroxybutyrate	B/A ratio
None	6.9 ± 0.4	37.0 ± 3.3	114 ± 13	64 ± 14	0.56 ± 0.08
S-15261					
0.1 mM	8.7 ± 0.5	29.1 ± 3.1	117 ± 14	39 ± 15	0.30 ± 0.08
1 mM	8.4 ± 1.2	29.9 ± 4.7	124 ± 10	56 ± 19	0.45 ± 0.05
S-15511					
0.1 mM	7.0 ± 0.4	33.1 ± 3.7	110 ± 13	45 ± 8	0.41 ± 0.06
1 mM	9.3 ± 1.0	35.3 ± 2.4	109 ± 13	55 ± 13	0.50 ± 0.05
Y-415					
0.1 mM	6.6 ± 0.4	30.9 ± 2.8	121 ± 14	43 ± 8	0.35 ± 0.08
1 mM	9.0 ± 1.6	26.7 ± 3.1	96 ± 9	46 ± 15	0.47 ± 0.07

Data are means ± S.E.M. 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 S-15261 or its metabolites at the indicated concentration. B/A: β-hydroxybutyrate/acetoacetate ratio; ASP: acid soluble products.

greater reduction in glucose production rates than in cells cultured in the presence of insulin alone. By contrast, such additional effect was not observed when hepatocytes were cultured in the presence of both insulin and Y-415 (Table 3).

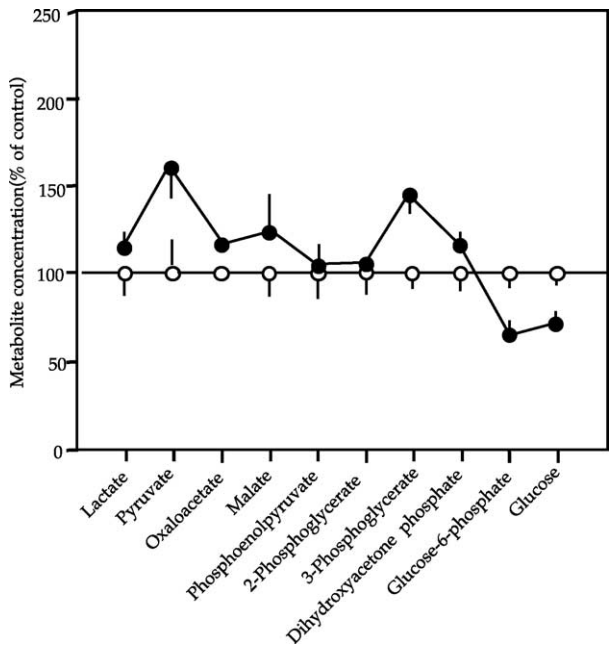

Fig. 3. Crossover plot showing the effect of Y-415 on the concentration of gluconeogenic intermediates in isolated hepatocytes incubated for 1 h in the presence of alanine 10 mM. The intermediate concentration in hepatocytes treated for 1 h in the presence of 0.1 mmol/l (●) was expressed as percent of that found in control hepatocytes (○). The concentration of each metabolite in control cells is given in nmoles per 10^6 hepatocytes: lactate = 62 ± 8; pyruvate = 18 ± 2; calculated oxaloacetate = 0.24; malate = 3.1 ± 0.6; phosphoenolpyruvate = 2.3 ± 0.4; 2-phosphoglycerate = 0.6 ± 0.2; 3-phosphoglycerate = 3.5 ± 0.6; dihydroxyacetone phosphate = 3.8 ± 0.7; glucose-6-phosphate = 0.52 ± 0.11; glucose = 164 ± 16. Results are means ± S.E.M. of six different experiments performed in duplicate.

3.5. Effect of S-15261 and its metabolites S-15511 and Y-415 on hepatic gene expression in cultured hepatocytes

In order to determine whether long-term effect on hepatic gluconeogenesis could be due to in part to changes in the expression of genes encoding regulatory protein involved in glucose metabolism (transport, glycolysis, gluconeogenesis and lipogenesis), the levels of mRNA encoding Glut-2, glucokinase (GK), liver type pyruvate kinase (L-PK), glucose-6-phosphatase (Glc-6-Pase), phosphoenolpyruvate carboxykinase (PEPCK) and fatty acid synthase (FAS) were determined in hepatocytes cultured

Table 3
Dose-dependent effect of S15261 and its metabolites on gluconeogenesis in cultured hepatocytes from 24 h starved rats

	Without insulin	Plus insulin
Control	446 ± 29	284 ± 19**
S 15261		
10 μM	317 ± 44**	242 ± 40
100 μM	200 ± 37**	184 ± 23$^{\$}$
S 15511		
10 μM	375 ± 20*	251 ± 32
100 μM	190 ± 23**	178 ± 14$^{\$}$
Y 415		
10 μM	328 ± 43*	233 ± 29
100 μM	235 ± 37**	210 ± 37

Hepatocytes from 24 h-starved adult rat were cultured for 48 h in the absence or in the presence of S 15261 or its two metabolites at the indicated concentrations and without or in the presence of insulin (10^{-10} M). Two hours before the end of the culture, the medium was replaced by a medium containing the same experimental conditions and a mixture of lactate/pyruvate (10/1 mM). The rates of glucose production were measured after the last 2 h of culture and expressed as nmol/2 h/mg of protein. Results are means ± S.E.M. of six different experiments performed in duplicate.

* $P < 0.05$ when compared to control.
** $P < 0.01$ when compared to control.
$^{\$}$ $P < 0.05$ when compared to control plus insulin.

24 h in the presence of each compounds either in the absence or the presence of insulin 10^{-10} M. As previously reported in vivo [11], neither S-15261 nor its metabolites affected the expression of Glut-2 or L-PK genes whatever the concentration used (10 or 100 μM, data not shown). By contrast, when used at the higher concentration (100 μM), S-15261 and its metabolites decreased the expression of gene encoding for the key gluconeogenic enzymes, PEPCK and Glc-6-Pase (Table 4). Interestingly, the profile of PGC-1 mRNA (a key coactivator involved in the regulation of PEPCK and Glc-6-Pase gene transcription [22,23]) followed the same pattern as PEPCK and Glc-6-Pase mRNA levels (in arbitrary units normalized to cyclophilin mRNA values: control = 89 ± 4; S-15261 100 μM = 55 ± 10*; S-15511 100 μM = 61 ± 8*; Y-415 = 40 ± 6*; n = 6–8; *$P < 0.05$ when compared to control). As already mentioned for hepatic glucose production, the inhibitory effects of S-15261 and S-15511 were more pronounced in the presence of insulin (Table 4) whereas Y-415 did not enhance the inhibitory effect of insulin on PEPCK and Glc-6-Pase mRNA levels (Table 4). Similarly, the inhibitory effect of S-15511 on PGC-1 gene expression was enhanced in the presence of insulin (in arbitrary units normalized to cyclophilin mRNA values: control + insulin = 72 ± 9; S-15511 100 μM + insulin = 44 ± 9; n = 6; *$P < 0.05$ when compared to control + insulin). Conversely, S-15511 increased by two- to four-fold the expression of GK gene in the absence or the presence of insulin, respectively (Table 4) whereas S-15261 and Y-415 had no effect on GK mRNA level (Table 4). Interestingly, the mRNA level of SREBP-1c, a key transcription factor involved in the stimulation GK gene expression by insulin [24], followed similar pattern of expression as GK gene (in arbitrary units normalized to cyclophilin mRNA values: control = 55 ± 7; control + insulin = 100 ± 1*; S-15511 100 μM = 230 ± 40*; S-15511 100 μM + insulin = 287 ± 45= n = 9; *$P < 0.05$ when compared to control; =$P < 0.05$ when compared to control + insulin). Neither S-15261 nor Y-415 affected the SREBP-1c mRNA levels (data not shown). Finally, S-15261 and its metabolites induced, in a dose dependent manner, the expression of FAS (Table 4). The presence of insulin in the culture medium did not increase the FAS mRNA level either in the absence or in the presence of either compounds (Table 4) despite the additional effect of insulin on SREBP-1c mRNA levels (see above).

4. Discussion

S-15261 is a new oral anti-hyperglycemic agent, which normalizes plasma glucose levels and reduces circulating concentrations of triglycerides and fatty acids [10,11,13,14]. The question addressed in this study was to know whether these beneficial effects on metabolism were due to S-15261 per se and/or to an insulin-mediated effect. The present work suggests that the decrease in free fatty acid concentration in the plasma of animal models treated with S-15261 seems not due to an accelerated fatty acid oxidation in the liver. Indeed, neither the rate of fatty acid oxidation nor the expression of gene encoding regulatory protein of this pathway (carnitine palmitoyltransferase I and mitochondrial hydroxymethylglutaryl-CoA synthase, data not shown) is affected by S-15261 or its metabolite. The fact that neither fatty acid oxidation nor ketogenesis is altered by S-15261 or its metabolites clearly demonstrates that the inhibitory effects of these compounds on hepatic glucose production are specific and not due to deleterious effects on isolated hepatocytes. Accordingly, our data suggests that normalization of blood glucose in insulin-resistant animal models treated with S-15261 [9–11] could be due, at least in part, to a reduction in hepatic gluconeogenesis through acute and chronic effects. During short-term exposure and for a given concentration, Y-415 seemed the more active metabolite of S-15261 on this pathway. Although the mechanism(s) by which Y-415

Table 4
Effect of S15261 and its metabolites on hepatic gene expression in cultured hepatocytes from 24 h starved rats

	PEPCK		Glc-6-Pase		GK		FAS	
	−Insulin	+Insulin	−Insulin	+Insulin	−Insulin	+Insulin	−Insulin	+Insulin
None	94 ± 4	41 ± 8*	89 ± 9	37 ± 9*	46 ± 9	101 ± 3*	101 ± 7	163 ± 28
S 15261								
10 μM	75 ± 17	29 ± 4	87 ± 17	27 ± 7	51 ± 20	74 ± 16	183 ± 49	187 ± 37
100 μM	40 ± 7*	12 ± 2†	47 ± 16*	15 ± 9	35 ± 7	57 ± 13	360 ± 86*	304 ± 56†
S 15511								
10 μM	87 ± 11	30 ± 4	82 ± 10	24 ± 6	51 ± 10	127 ± 37	151 ± 40	185 ± 39
100 μM	33 ± 11*	18 ± 5†	31 ± 9*	12 ± 5†	103 ± 18*	192 ± 45†	534 ± 146*	554 ± 127†
Y 415								
10 μM	66 ± 17	28 ± 5	90 ± 20	22 ± 6	67 ± 13	74 ± 15	167 ± 14	179 ± 36
100 μM	50 ± 10*	22 ± 6	54 ± 10*	19 ± 8	33 ± 8	46 ± 7	301 ± 64*	283 ± 49†

Total RNA were extracted from 24 h-starved adult rat hepatocytes cultured in the absence or in the presence of insulin 10^{-10} M and S 15261 or its two metabolites at the indicated concentrations. mRNA level were determined by real time quantitative PCR using primers listed in Table 1 and were normalized to cyclophilin mRNA values. Results are means ± S.E.M. of six to nine different experiments performed in duplicate.

* $P < 0.05$ when compared to control cell (−insulin).

† $P < 0.05$ when compared to hepatocytes cultured in the presence of insulin.

reduced hepatic glucose production is (are) still unknown, our data demonstrated that the reduction of gluconeogenesis by Y-415 was localized between triose phosphate and glucose. These conclusions were reinforced by the fact that (1) Y-415 failed to affect the cytosolic redox state, a process that controls gluconeogenic steps downstream dihydroxyacetone phosphate synthesis [17] and (2) glucose production was markedly reduced from dihydroxyacetone a gluconeogenic substrate that enters the pathway downstream the reaction catalyzed by glyceraldehydes-3-phosphate dehydrogenase. At least three other anti-diabetic agents showed such kind of inhibitory effect: metformin [25], troglitazone [20] and glibenclamide [26]. Metformin decreased the activity of glucose-6-phosphatase [27] whereas both troglitazone and glibenclamide induced an increase in fructose-2,6-bisphosphate concentration, a potent inhibitor of fructose-1,6-bisphosphatase [26,28]. It is noteworthy that the effect of glibenclamide on fructose-2,6-bisphosphate concentration was not due to its non-sulfonylurea moiety, meglitinide (4-[2-(5-choloro-2-methoxybenzamide)ethyl]-benzoic acid), that induced a decrease in fructose-2,6-bisphosphate concentration rather than an increase [26]. As Y-415 is related to this family of benzoic acid derivatives, which also stimulates insulin secretion and present anti-hyperglycemic properties, it could be emphasized that its mechanism of action on hepatic gluconeogenesis should be similar. These insulin-independent effects of S-15261 and its metabolites are also observed during longer-term exposure to more ''physiologic'' concentration of these drugs. Indeed, S-15261, S-15511 and Y-415 decrease hepatic gluconeogenesis in an insulin-independent manner as a result of an inhibition of PEPCK and Glc-6-Pase gene expression. Moreover, S-15261 through its metabolite S-15511 enhances the inhibitory effect of insulin on PEPCK and Glc-6-Pase mRNA levels and on hepatic glucose production rates. The direct effect of S-15261 and its metabolites on PEPCK and Glc-6-Pase gene expression could be due, in part, to the drug-induced reduction in PGC-1 gene expression. It has been shown that this transcriptional coactivator could contribute to hepatic insulin resistance through an up-regulation of PEPCK and Glc-6-Pase gene transcription [22,23]. Indeed, disruption of hepatic PGC-1 expression induces marked fall in fasting plasma glucose concentration and PEPCK or Glc-6-Pase mRNA levels in deficient mice liver [29]. Altogether these results showed that S-15261 could reduce hyperglycemia through an inhibition of hepatic glucose production via direct and insulin-sensitizing effects thus confirming in a more direct way, the data from in vivo experiments [11].

The other way to decrease blood glucose level would be to enhance glucose utilization not only in peripheral tissues as already demonstrated in vivo with S-15261 [13,14] but also in the liver. Indeed, it was shown that S-15261 induced a stimulation of glucokinase gene expression in the liver of JCR:LA-cp rats treated daily with 30 mg/kg of this compound [11], suggesting that S-15261 should affect glycolysis and gluconeogenesis in an opposite way. Interestingly, the metabolite S-15511 is able to enhance the expression GK gene in the absence of insulin as a result of a stimulatory effect on SREBP-1c gene expression. Moreover, S-15511 enhances the stimulatory effect of insulin on GK mRNA level through an additional increase in SREBP-1c gene expression. These results are in agreement with the key role of SREBP-1c in the regulation of GK gene transcription (reviewed in: [30]). Despite a marked rise in SREBP-1c gene expression, S-15261 and its metabolites are not able to increase liver-type pyruvate kinase (L-PK) either in the absence or in the presence of insulin. This lack of L-PK gene induction was already observed in the liver of JCR:LA-cp rats treated with S-15261 [11]. As hepatocytes are cultured in the absence of glucose, this would suggest that the induction of L-PK gene is more dependent on glucose metabolism than on SREBP-1c expression as previously reported in cultured rat [31] and mice hepatocytes [32]. By contrast, fatty acid synthase mRNA levels are markedly induced by S-15261 and its metabolites as a consequence of the increase in SREBP-1c gene expression. These results are in agreement with the stimulatory effect of SREBP-1c overexpression on FAS gene in the liver of fasted mice or in cultured mice hepatocytes [32]. Indeed, it was shown in the liver of JCR:LA-cp rats that S-15261 stimulated the expression not only of fatty acid synthase, but also acetyl-CoA carboxylase, another regulatory enzyme in the synthesis of fatty acid from glucose [11].

The overall data are consistent with in vivo studies showing that S-15261, *via* its metabolite S-15511, improves insulin sensitivity. Moreover, the present work provides evidence that in addition to this insulin-sensitizing effect, this compound through its metabolites has specific effect on hepatic glucose metabolism either during short-term exposure (mainly *vi*a Y-415) or during chronic exposure secondary to changes in the expression of key transcriptional factor (SREBP-1c) or coactivator (PGC-1). These metabolic and genetic effects contribute to reduce hepatic glucose production and hyperglycemia, which in turn would improve peripheral insulin resistance.

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