Pharmacodynamic profile of a novel inhibitor of the hepatic glucose-6-phosphatase system

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Herling, Andreas W, Hans-Joerg Burger, Dietmar Schwab, Horst Hemmerle, Peter Below, and Gerrit Schubert. Pharmacodynamic profile of a novel inhibitor of the hepatic glucose-6-phosphatase system. Am. J. Physiol. 274 (Gastrointest. Liver Physiol. 37): G1087–G1093, 1998.—The glucose-6-phosphatase (G-6-Pase) system catalyzes the terminal enzymatic step of gluconeogenesis and glycogenolysis. Inhibition of the G-6-Pase system in the liver is expected to result in a reduction of hepatic glucose production irrespective of the relative contribution of gluconeogenesis or glycogenolysis to hepatic glucose output. In isolated perfused rat liver, S-3483, a derivative of chlorogenic acid, produced concentration-dependent inhibition of gluconeogenesis and glycogenolysis in a similar concentration range. In fed rats, glucagon-induced glycogenolysis resulted in hyperglycemia for nearly 2 h. Intravenous infusion of 50 mg·kg⁻¹·h⁻¹ S-3483 prevented the hyperglycemic peak and subsequently caused a further lowering of blood glucose. In 24-h starved rats, in which normoglycemia is maintained predominantly by gluconeogenesis, intravenous infusion of S-3483 resulted in a constant reduction of blood glucose levels. Intrahepatic concentrations of glucose-6-phosphate (G-6-P) and glycogen were significantly increased at the end of both in vivo studies. In contrast, lowering of blood glucose in starved rats by 3-mercaptopicolinic acid was accompanied by a reduction of G-6-P and glycogen. Our results demonstrate for the first time in vivo a pharmacologically induced suppression of hepatic G-6-P activity with subsequent changes in blood glucose levels.

inhibition of gluconeogenesis; inhibition of glycogenolysis; inhibition of hepatic glucose output; blood glucose reduction

GLUCOSE-6-PHOSPHATASE (G-6-Pase) (EC 3.1.3.9) has important functions in glucose metabolism and its homeostasis. G-6-Pase converts glucose-6-phosphate (G-6-P) into glucose and phosphate and represents 1) the terminal enzymatic step of hepatic and renal glucose production and 2) the common enzymatic step for both glucose-producing pathways, gluconeogenesis and glycogenolysis (6, 21).

Postabsorptively, endogenous glucose production by gluconeogenesis is essential to maintain normal blood glucose levels. Postprandially, glucose is mainly stored as glycogen in the liver, which can be mobilized and released as glucose very quickly if required. Mutations in the G-6-Pase gene that result in the complete absence of enzymatic activity have been identified in humans as the molecular cause for glycogen storage disease (GSD) type Ia (9). GSD is characterized by hypoglycemia, increased serum lactate levels, and excessive glycogen storage in the liver. The corresponding animal model, the G-6-Pase knockout mouse (19), showed similar characteristics with the exception that plasma lactate levels were not elevated.

G-6-P hydrolysis requires the coupled function of at least three integral proteins of the endoplasmic reticulum (ER): 1) the enzyme, G-6-Pase, with its catalytic site facing the lumen of the ER; 2) a G-6-P translocase, denoted T1; and 3) a second translocase, denoted T2, that mediates efflux of Pi (1, 4, 5).

The natural product chlorogenic acid (CHL) has recently been shown to be a specific competitive inhibitor of the G-6-P translocase. CHL inhibited G-6-P hydrolysis only in intact liver microsomes, but was without effect on 1) the inorganic pyrophosphatase activity of the system, thus excluding T2 as the site of inhibition, or 2) the G-6-P phosphohydrolase component of the system (1). The structure-activity relationship for these T1 inhibitors has been studied for a number of synthetic derivatives of CHL (3, 15). One of these, S-3483, is a highly potent competitive inhibitor of the G-6-Pase systems from the liver and kidneys (2).

The present study was performed to characterize pharmacologically the inhibitory profile of S-3483. The influence of S-3483 was examined in vitro in isolated perfused rat livers and in vivo on blood glucose levels in rats and compared with the inhibitory profile of 3-mercaptopicolinic acid (3-MPA), an inhibitor of gluconeogenesis at the level of phosphoenolpyruvate carboxykinase (PEPCK; EC 4.1.1.32) (14).

MATERIALS AND METHODS

Test compound S-3483. S-3483 (1-[2-(4-chlorophenyl)cyclopropyl]methoxy-3,4-dihydroxy-5-[3-(4-hydroxyphenyl)-1-oxo-2-propenyl]oxy-cyclohexanecarbonylic acid) has the empirical formula C₂₆H₂₇ClO₈ with a molecular weight of 502.95. Its molecular structure is shown in Fig. 1. 3-MPA was used as a reference compound. Both compounds were synthesized by the Chemistry Department at Hoechst Marion Roussel Deutschland GmbH.

For liver perfusion studies, compounds were dissolved in 1 ml DMSO and added to the medium at time 0. The concentrations indicated in RESULTS are calculated from the amounts of S-3483 or 3-MPA added to 100 ml (total vol) of the perfusion medium per liver. For intravenous formulation, S-3483 was dissolved in phosphate buffer, pH 7.4, containing 15% DMSO. 3-MPA was administered to rats intraperitoneally in a 1% (wt/vol) starch suspension.

Animals. Sprague-Dawley (Moellegard, Denmark; 250- to 300-g body wt) and Wistar (HoeWISKf SPF 71; 230- to 250-g body wt) rats were used for the experiments. They were housed in groups of up to five per cage in a temperature-controlled room with a 12:12-h light-dark cycle. All animals had free access to water and a standard pellet rat chow (Altromin 1320), unless otherwise indicated.

Isolated perfused rat livers. The procedure was performed as described previously (24) with the following specifications. Male Sprague-Dawley rats were anesthetized with pentobarbital sodium (60 mg/kg ip). The liver was exposed by longitudi-
dinal midline and transverse subcostal incisions, and the portal vein was cannulated with a venous cannula. The liver was immediately infused at 37°C with oxygenated saline containing heparin (70 U/ml). The vena cava caudalis was opened to allow a continuous flow of the saline and heparin solution for ~2 min. Then the liver was transferred into a heated (37°C) perfusion chamber and perfused via the portal vein in a recirculating manner at a constant flow rate of 35 ml/min with continuously oxygenated Krebs-Ringer-bicarbonate buffer (100 ml total vol). The buffer consisted of (in mmol/l) 137 NaCl, 2.7 KCl, 11.9 NaHCO₃, 0.72 NaH₂PO₄, 1.8 CaCl₂, and 0.5 MgCl₂ without glucose and was supplemented with 30% (vol/vol) washed bovine erythrocytes and 1.6% (wt/vol) BSA. Experiments were routinely carried out on four livers simultaneously. Samples of the perfusate were taken in 10-min intervals for the determination of glucose and lactate and every 30 min for determination of lactate dehydrogenase activity (LDH). The reported values for glucose, lactate, and LDH are cumulative values during a perfusion of up to 2 h. Glucose and lactate concentrations as well as LDH activity in the perfusate were determined by standard enzymatic procedures (7).

Inhibition of gluconeogenesis was tested in livers from rats starved for 24 h with free access to water. Hepatic glucose production from gluconeogenesis was stimulated by adding fructose or a mixture of lactate and pyruvate to the perfusate 30 min after the start of perfusion. Fructose was used at a final concentration of 10 mmol/l, and lactate and pyruvate were used in a mixture at concentrations of 20 and 2 mmol/l, respectively. The test compounds were added to the perfusate at the beginning of the perfusion. S-3483 was studied at concentrations ranging from 0.01 to 1 mmol/l, and 3-MPA was studied at a single concentration of 1 mmol/l.

Inhibition of glycogenolysis was tested in livers from rats with free access to food and water before the beginning of the experiment. S-3483 was added to the perfusate at the start of the experiment at concentrations ranging from 0.03 to 1 mmol/l.

Blood glucose profile in rats. Blood glucose levels were assayed in anesthetized male Wistar rats using a modified method of glucose clamp studies in rodents (22). Rats were anesthetized with an intraperitoneal injection of pentobarbital sodium (60 mg/kg) and tracheotomized, and one jugular vein per rat was cannulated for intravenous infusion. Anesthesia was maintained for up to 7 h by subcutaneous infusion of pentobarbital sodium (adjusted to the anesthetic depth of the individual animal; ~24 mg·kg⁻¹·h⁻¹). Body temperature was monitored with a rectal probe thermometer, and temperature was maintained at 37°C by means of a heated surgical table. Blood samples (10 µl) were obtained from the tip of the tail every 15 min for glucose analysis and every 30 min for lactate analysis (20 µl). We allowed blood glucose levels in the rats to stabilize after surgery for up to 2 h. S-3483 was then infused intravenously at a dose of 50 mg·kg⁻¹·h⁻¹ for a period of 5 h. 3-MPA was administered intraperitoneally twice at doses of 100 and 25 mg/kg, respectively; the second dose was administered 3 h after the first one to obtain a blood glucose-lowering effect that was comparable in its duration to that obtained with S-3483.

At the end of the experiment, the abdomen was opened, and a part of the liver was freeze-dried immediately. The frozen tissue was stored in liquid nitrogen for subsequent determinations of intrahepatic concentrations of glycogen and G-6-P. Standard enzymatic procedures were used to determine glucose, lactate, glycogen, and G-6-P (7).

To study the effect of a test compound on the process of gluconeogenesis, rats were starved for 24 h, because under these conditions euglycemia is maintained exclusively by gluconeogenesis due to the very low glycogen content in the liver. In contrast, to investigate the effect of the test compound on the process of glycogenolysis, rats had free access to food until the start of the experiment. During this study, glycogenolysis was induced by an intravenous bolus injection of glucagon at a dose of 1 mg/rat. It can be assumed that the hyperglycemia induced by the glucagon injection, which lasted for ~90–120 min, was the result of the glucagon-induced breakdown of hepatic glycogen.

Statistics. Results are presented as means ± SE. An estimate of I₅₀ values in isolated perfused liver studies was obtained graphically. I₅₀ values refer to the calculated concentrations at time 0 when the compounds were added to 100 ml of perfusion medium. Statistical differences (P < 0.05) were assessed by Student’s t-test; n indicates the number of animals.

RESULTS

Typical morning blood glucose levels were in the range of 4–5 mmol/l in 24-h starved animals and 6–7 mmol/l in fed rats. Liver glycogen content was ~25 µmol/g and between 200 and 300 µmol/g in starved and fed rats, respectively. The dramatic reduction of hepatic glycogen stores in rats starved for 24 h confirms the well-known fact that in such animals the fasting blood
glucose level of 4–5 mmol/l is maintained predominantly by the process of gluconeogenesis. Because no glucose was present in the medium at the beginning of the perfusion experiment, that which appeared during perfusion must have been produced by the liver itself. Endogenous hepatic glucose production by isolated livers from starved rats was low (22.6 ± 3.9 µmol glucose/g liver after 2 h of perfusion). However, when fructose was added to the perfusate at a final concentration of 10 mmol/l it was almost completely transformed into glucose during the perfusion period of 2 h (Fig. 2). This glucose production process essentially indicates the functional integrity and viability of the livers during the perfusion period in our experimental setup. LDH released into the perfusate, which did not significantly differ between livers perfused in the absence or presence of the test compounds (data not shown), further indicates that there was no compound-induced damage of the livers that could also result in reduced hepatic glucose production.

Influence of S-3483 on gluconeogenesis. S-3483 caused a concentration-dependent decrease of fructose-stimulated hepatic glucose output in isolated perfused livers of fasted rats with an estimated IC50 of ~100 µmol/l. In parallel, hepatic lactate production increased, reaching maximal values that showed a comparable concentration dependency (Fig. 2). Subsequently, lactate levels declined gradually (1, 0.3, and 0.1 mmol/l S-3483) or nearly returned to initial values (0.03 and 0.01 mmol/l S-3483 and control). When we used lactate and pyruvate as gluconeogenic precursors, S-3483 caused a similar decrease in hepatic glucose output (data not shown). In contrast, the activity profile was markedly different when we studied the effects of 3-MPA, a well-described inhibitor of gluconeogenesis, at a concentration of 1 mmol/l, using fructose or lactate and pyruvate as gluconeogenic precursors at the same concentrations as before. 3-MPA treatment abolished glucose production stimulated by lactate and pyruvate and only modestly decreased gluconeogenesis from fructose (Fig. 3).

Influence of S-3483 on glycogenolysis. The increase of glucose in the perfusate during the perfusion studies of livers from fed rats without any substrate in the medium was derived from glycogenolysis, the breakdown of hepatic glycogen to glucose that subsequently is released into the perfusate. Addition of S-3483 to the
perfusate resulted in a concentration-dependent decrease of hepatic glucose output under this condition (Fig. 4). The inhibitory potency of the compound was similar to that observed in livers from starved rats. However, hepatic lactate production did not show the concentration-dependent increase observed in livers from starved rats. In contrast, 3-MPA at 1 mmol/l did not have any inhibitory effect on glucose production in livers from fed rats (Fig. 3). Taken together, these findings in isolated perfused livers are consistent with a common site for the inhibition of gluconeogenesis and glycogenolysis by S-3483.

Glucose-lowering action of S-3483 in vivo. Having established that S-3483 can inhibit hepatic glucose production in isolated perfused rat livers with similar potency irrespective of the metabolic pathways predominantly involved in the process, we investigated the effects of S-3483 in rats in vivo. S-3483 was administered as a constant intravenous infusion into anesthetized rats. In fed rats, infusion of 50 mg·kg⁻¹·h⁻¹ S-3483 resulted in a decrease of blood glucose even when glycogenolysis was induced by intravenous glucagon (Fig. 5). S-3483 prevented the appearance of the glucagon-induced hyperglycemic peak observed in control rats after glucagon injection and subsequently caused a further reduction in blood glucose. Liver glycogen and G-6-P, determined at the end of the study, were significantly higher in livers from treated fed rats compared with livers from control rats (Table 1).

In starved rats, in which normoglycemia (4–5 mmol/l) is primarily maintained by the process of gluconeogenesis, infusion of 50 mg·kg⁻¹·h⁻¹ S-3483 resulted in a rapid reduction of the blood glucose level over the first 90 min of infusion, after which the blood glucose level remained nearly constant (Fig. 6). At the end of the study, blood glucose was significantly decreased, while blood lactate, hepatic glycogen, and hepatic G-6-P concentrations were significantly increased (Table 1).

<table>
<thead>
<tr>
<th>Assay Condition</th>
<th>Test Compound</th>
<th>Blood Glucose, mmol/l</th>
<th>Blood Lactate, mmol/l</th>
<th>Glycogen, µmol/g</th>
<th>G-6-P, nmol/g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fed + glucagon</td>
<td>Control</td>
<td>5.7 ± 0.3</td>
<td>1.0 ± 0.1</td>
<td>30 ± 9</td>
<td>247 ± 42</td>
</tr>
<tr>
<td></td>
<td>S-3483</td>
<td>3.8 ± 0.4*</td>
<td>1.9 ± 0.2*</td>
<td>83 ± 18*</td>
<td>471 ± 84*</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>3.7 ± 0.2</td>
<td>1.6 ± 0.3</td>
<td>22 ± 7</td>
<td>59 ± 14</td>
</tr>
<tr>
<td></td>
<td>S-3483</td>
<td>1.8 ± 0.4*</td>
<td>4.6 ± 1.5*</td>
<td>66 ± 17*</td>
<td>106 ± 10*</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>4.3 ± 0.3</td>
<td>1.5 ± 0.3</td>
<td>16 ± 4</td>
<td>98 ± 10</td>
</tr>
<tr>
<td></td>
<td>3-MPA</td>
<td>0.9 ± 0.2*</td>
<td>3.6 ± 0.3*</td>
<td>0.4 ± 0.1*</td>
<td>12 ± 1*</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 5–6. Data shown are from rat livers at the end of experiments shown in Figs. 5–7. G-6-P, glucose-6-phosphate. 3-MPA, 3-mercaptopicolinic acid. *P < 0.05.
3-MPA was also used in vivo as a reference compound to reduce hepatic glucose output by inhibition of gluconeogenesis in starved rats. For comparison of the effects of 3-MPA with those of S-3483 on hepatic glycogen and G-6-P levels, we attempted to develop a protocol for compound administration that would result in a blood glucose profile similar to that obtained during S-3483 intravenous infusion. In our study, the dose of 3-MPA necessary to reduce blood glucose levels could not be administered by intravenous infusion due to solubility problems. Therefore, we next administered 3-MPA at a dose of 100 mg/kg intraperitoneally, which reduced blood glucose levels only for ~3 h. Subsequently, blood glucose levels recovered nearly to control values (data not shown). Only when we administered 3-MPA as an initial dose of 100 mg/kg intraperitoneally followed by a second dose of 25 mg/kg 3 h later were we able to obtain a blood glucose profile similar to that during S-3483 intravenous infusion (Fig. 7). However, in contrast to the increase of liver glycogen and G-6-P seen after S-3483 treatment, both parameters were significantly reduced after 3-MPA treatment, although the blood glucose profile was similarly reduced over a period of 5 h (Table 1).

**DISCUSSION**

S-3483 inhibited glucose production in isolated perfused rat livers irrespective of the metabolic pathway that predominantly contributed to hepatic glucose output in our experimental setup. This finding is in accordance with predictions for inhibition of a metabolic site that is common to the glucogenic pathways of gluconeogenesis and glycogenolysis. The marginally lower potency for inhibition of glycogenolysis by S-3483 in perfused rat livers most likely reflects the fact that the CHL derivatives inhibit competitively with respect to the substrate G-6-P (1, 2). Therefore, a higher concentration of inhibitor was necessary to achieve the same degree of inhibition under conditions of glycogenolysis, in which hepatocellular G-6-P concentrations were significantly higher than in the gluconeogenic condition (Table 1). The transiently increased lactate levels observed in the control group of fasted rats probably reflect partial glycolytic conversion of fructose to pyruvate, which then serves as an additional gluconeogenic substrate ("indirect pathway"; see Ref. 20). The lactate profile of the treated groups, especially at higher inhibitor concentrations, does not most likely stem from compound inactivation by the liver but rather indicates a pronounced redirection of carbon flux from gluconeogenesis to glycolysis in our perfusion experiments.

The experiments in rats were performed to explore the pharmacodynamic properties of S-3483 in vivo. The basic observations from liver perfusion studies could also be demonstrated in vivo in anesthetized rats. In starved rats, blood glucose levels are maintained predominantly by the process of gluconeogenesis. Intravenous infusion of S-3483 lowered blood glucose in starved rats, which we interpret to be at least in part the inhibitory effect of the compound on the process of gluconeogenesis as seen in perfused livers from starved rats. Furthermore, S-3483 also interfered with glycogenolysis in vivo, most evidently demonstrated by the ablation of the hyperglycemia observed in response to the glucagon-induced breakdown of liver glycogen in control animals. The hepatic content of glycogen and G-6-P was substantially increased after S-3483 treatment. The simultaneous depression of blood glucose with an increase in the concentrations of hepatic G-6-P and glycogen was observed irrespective of the glucogenic pathways and was seen only after S-3483 infusion. These responses are exactly what is expected for a competitive inhibitor of G-6-Pase.

The inhibitory profile for S-3483 can be contrasted with that obtained for 3-MPA, a well-known inhibitor of only gluconeogenesis at the level of PEPCK (14, 20). In isolated perfused rat livers from starved rats, 3-MPA inhibited hepatic glucose production induced by lactate and pyruvate much more effectively than gluconeogenesis induced by fructose. Although it could be argued that the modest inhibitory effect on the latter is the result of an additional inhibitory action of 3-MPA on...
G-6-Pase activity at the millimolar concentration used in this study (8, 13), it must be noted that in livers from fed rats, we did not observe any inhibitory effect on glucose output derived from glycogenolysis. Thus these data are consistent with an inhibitory effect of 3-MPA only at the level of PEPCK. This conclusion is also supported by the influence of 3-MPA in starved rats on liver metabolites, particularly the reduced concentrations of both G-6-P and glycogen, indicating that inhibition was upstream from the site of G-6-P hydrolysis.

With respect to the observations of blood glucose changes in starved rats in vivo after treatment with test compounds, it should be noted that not only the liver but also the kidney has a significant function in blood glucose homeostasis, especially regarding the process of gluconeogenesis during starvation. Both organs possess all the key gluconeogenic enzymes in sufficient amounts to significantly contribute to total glucose production. Renal glucose production and utilization can account for up to 30% of glucose turnover in postabsorptive dogs (11). Therefore, the observed effects on blood glucose in vivo after systemic administration of S-3483 and 3-MPA to rats reflect the sum of the effects on net glucose production by the liver and kidneys. Thus it must be noted that the CHL analog S-3483 inhibits G-6-P hydrolysis in intact microsomes isolated from rat liver or kidney cortex with similar potency (2).

Taken together, the observed effects of the CHL derivative S-3483 on isolated perfused rat livers, as well as in vivo in rats, strongly suggest that the effects of the compound are due to the same mechanism of action in the intact organ and also in the whole animal in vivo that has been defined for the natural product CHL (1) and for its derivatives with isolated microsomes in vitro (2, 15), the inhibition of the G-6-Pase system by blocking G-6-P binding to the G-6-P translocating component. Further evidence for this conclusion is found in the comparison of our results with the well-known changes of metabolic parameters in GSD type I patients (10). Pathophysiologically, in GSD type I patients, the absence of functional G-6-Pase activity results in hypoglycemia, hyperlactatemia, and excessive glycogen storage in the liver. This characteristic profile is similar to that reported here for treatment of animals with S-3483.

In non-insulin-dependent diabetes (NIDDM), increased fasting blood glucose levels result from increased endogenous glucose production (12). Even relatively normal rates of endogenous glucose production are inappropriate in relation to the increased fasting blood glucose levels generally seen in NIDDM (16, 17). It has been difficult in the past to influence liver total hepatic glucose output in humans solely by interfering with the gluconeogenic pathway (18, 25). This was explained by a functioning autoregulatory mechanism in the liver that increases the release of glucose from glycogen as long as hepatic glycogen is still available (18). Inhibition of hepatic glucose output by interference with gluconeogenesis might function only under conditions of extreme fasting, when glycogen stores are fully depleted. Inhibition of the G-6-Pase system, the terminal step of endogenous glucose production, is the most effective way to affect hepatic glucose output irrespective of the nutritional state. In addition, inhibition of endogenous hepatic glucose production at the level of G-6-Pase should also reduce the putative risk of lactic acidosis, relative to blocks imposed within the gluconeogenic pathways, because under inhibitory conditions a redirection of the carbon flow into glycogen is possible. Indeed, this was demonstrated by the increase in liver glycogen at the end of the experiments with both starved and fed rats in our study. In addition to a pure mass effect increased levels of G-6-P, activation of liver glycogen synthase could in part contribute to a redirection of the metabolic flux from G-6-P hydrolysis toward the synthesis of glycogen. Recently, it has been found that G-6-P can activate liver glycogen synthase b (23).

For better treatment of NIDDM, one could envision pharmacologically modulated reduction of G-6-Pase activity to normalize the inappropriately high rates of hepatic glucose production. A therapeutic regimen would not, however, attempt to cause hypoglycemia as we have tried to achieve here to provide proof of the concept that the pharmacodynamic activity of S-3483 acts as an inhibitor of the G-6-Pase system.

In conclusion, CHL analogs could become useful tools 1) to study GSD experimentally by pharmacological induction of metabolic changes in rats that are characteristic of this genetic disease and 2) to investigate the therapeutic potential of a new approach to the treatment of NIDDM.

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