The Flavonoid Silibinin Decreases Glucose-6-Phosphate Hydrolysis in Perfused Rat Hepatocytes by an Inhibitory Effect on Glucose-6-Phosphatase

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Key Words

Silibinin • Flavonoid • Hepatocytes • Gluconeogenesis • Glucose-6-phosphatase • Type 2 diabetes

Abstract

Background/Aims: The flavonoid silibinin has been reported to be beneficial in several hepatic disorders. Recent evidence also suggests that silibinin could be beneficial in the treatment of type 2 diabetes, owing to its anti-hyperglycemic properties. However, the mechanism(s) underlying these metabolic effects remains unknown. **Methods:** The effects of silibinin on liver gluconeogenesis were studied by titrating hepatocytes from starved rats with sub-saturating concentrations of various exogenous substrates in a perfusion system. Hepatocytes from fed rats were also used to investigate glycogenolysis from endogenous glycogen. The effect of silibinin on glucose-6-phosphatase kinetics was determined in intact and permeabilized rat liver microsomes. **Results:** Silibinin induced a dose-dependent inhibition of gluconeogen-

esis associated with a potent decrease in glucose-6-phosphate hydrolysis. This effect was demonstrated whatever the gluconeogenic substrates used, *i.e.* dihydroxyacetone, lactate/pyruvate, glycerol and fructose. In addition, silibinin decreased the glucagon-induced stimulation of both gluconeogenesis and glycogenolysis, this being associated with a reduction of glucose-6-phosphate hydrolysis. Silibinin inhibits glucose-6-phosphatase in rat liver microsomes in a concentration-dependent manner that could explain the decrease in glucose-6-phosphate hydrolysis seen in intact cells. **Conclusion:** The inhibitory effect of silibinin on both hepatic glucose-6-phosphatase and gluconeogenesis suggests that its use may be interesting in treatment of type 2 diabetes.

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Introduction

Flavonoids are the most abundant polyphenolic compounds present in the human diet, with a daily consumption estimated at 1 g [1]. Thousands flavonoids have been identified in fruits, vegetables and plant-derived

beverages, such as tea and red wine, and have been also associated with a wide variety of biochemical and pharmacological properties beneficial to health. Silymarin, one of the oldest traditional herbal medicines, is a lipophilic extract from the milk thistle *Silybum marianum* which is predominantly composed of four flavonolignan isomers: silibinin, isosilibinin, silidianin and silichristin. Silibinin constitutes 50-70% of the silymarin mixture and has been identified as the major biologically active component [2].

While for centuries extracts of milk thistle have been empirically used to combat multiple organ disorders, a growing body of evidence shows that silibinin could be clinically efficient in several hepatic diseases, such as cirrhosis or acute and chronic viral hepatitis [2-4]. The most remarkable use of silibinin (Legalon®) remains in the treatment of acute *Amanita phalloides* mushroom poisoning where its hepatoprotective effects after accidental ingestion have been demonstrated repeatedly in humans [4]. In addition, silibinin has been reported to protect the liver against toxicity induced by a wide range of chemical and environmental agents. A large part of these effects are attributed to its antioxidant properties (see [4] for review). Silibinin also received attention because of other effects not directly related to its antioxidant properties, *i.e.* the flavonoid exerting potential anti-carcinogenic [5, 6], anti-inflammatory [7] or neuroprotective [8] activities. Finally, silibinin has been recently proposed to be beneficial in type 2 diabetes since treatment with an oral formulation induced a significant decrease in both fasting and mean daily glycemia [9-11] and in HbA_{1c}, triglyceride and total cholesterol levels [11] in patients. Furthermore, aqueous extract of *Silybum marianum* exhibits potent hypoglycaemic and anti-hyperglycaemic activities in both streptozotocin- [12] and alloxan-induced diabetic rats [13]. The underlying mechanism could be related to an inhibition of hepatic glucose production but remains unknown to date.

Glucose-6-phosphatase (G6Pase) is associated with the endoplasmic reticulum and catalyses the reaction of glucose-6-phosphate (Glc-6-P) hydrolysis [14, 15]. The particular location of this enzyme at the crossroads of multiple pathways generating Glc-6-P confers a central role on G6Pase, notably in the regulation of hepatic glucose production [16]. While regulation of G6Pase gene expression is well characterised [17], a post-transcriptional regulation of the enzyme is still a matter of debate. Indeed, before the first demonstration of a short-term effect on G6Pase kinetics [18], it was commonly thought that enzyme activity was exclusively modulated by intracellular Glc-6-P levels. Other acute changes in G6Pase activity

were then reported, mediated by either insulin, metabolites such as proline or α -ketoglutarate, or fatty acids (see [19] for review). In addition, we have recently demonstrated that acute stimulation of gluconeogenesis by glucagon could be also mediated, at least in part, by a temperature-sensitive activation of Glc-6-P hydrolysis [20].

In the present study we investigated the metabolic effects of silibinin in rat hepatocytes perfused with different carbohydrates, and more particularly on gluconeogenic pathway by focusing on Glc-6-P hydrolysis at the level of G6Pase.

Materials and Methods

Hepatocytes isolation

Liver cells were prepared between 8.00 and 10.00 am from fed or 24 h-starved male Wistar rats (200-300 g). Animals were anaesthetized with intraperitoneal injection of sodium thiopental (125 mg.kg⁻¹) and hepatocytes were isolated in the presence of collagenase according to the method of Berry and Friend [21] modified by Groen et al. [22]. All procedures were performed in accordance with the principles and guidelines established by the European Convention for the Protection of Laboratory Animals.

Perfusion of hepatocytes

Hepatocytes from 24 h-starved rats (200-250 mg dry cells) were perfused at 37°C with Krebs-bicarbonate-calcium (120 mM NaCl, 4.8 mM KCl, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 24 mM NaHCO₃, 1.3 mM CaCl₂, pH 7.4) saturated with O₂/CO₂ (19:1) at a flow rate of 5 ml.min⁻¹, according to the method of Van der Meer and Tager [23] as modified by Groen et al. [22]. After 35 min, when a steady state has been reached, the cells were titrated with increasing substrate concentrations (0.15, 0.3, 0.6, 1.2, 2.4 and 4.8 mM) and in the presence of 25 or 100 μ M silibinin, 10⁻⁷ M glucagon or vehicle, alone or in combination. At the end of each steady state of 15 min, extracellular medium ("perfusate") and cell samples were collected at the exit or inside the perfusion chamber, respectively. For the time-course experiments, hepatocytes from fed rats (120 mg dry cells) were perfused as described above without addition of exogenous substrate and in the presence of 100 μ M silibinin or vehicle. 10⁻⁷ M glucagon was added in both chambers after 25 min and perfusate was collected at the indicated time. Cell samples were also removed from the chamber for determination of intracellular Glc-6-P level at the indicated time.

Determination of metabolic fluxes and intracellular intermediates and adenine nucleotides concentrations

The proteins in perfusate were denatured by heating in a warm water bath (80°C for 10 min) and glucose, lactate and pyruvate concentrations were then measured by spectrophotometric assays [20]. The net rates of glucose (J_{Glucose}) or lac-

tate-plus-pyruvate production (J_{L+P}) were calculated from the total cell content of the perfusion chamber, the flow rate through the system and the concentration of each metabolite in the perfusate, and were expressed as $\mu\text{moles} \cdot \text{min}^{-1} \cdot \text{g}$ dry cells $^{-1}$. The total metabolic flux from exogenous substrate ($J_{\text{Substrate}}$) was expressed as three-carbon equivalents and calculated from the sum of $2 \times J_{\text{Glucose}}$ and J_{L+P} . Once removed from the chamber, the cell sample was centrifuged through a layer of silicone oil (Rhodorsil 640 V 100) into HClO_4 (10% mass/vol) to separate intracellular content from extracellular medium. The acid-soluble fraction was then neutralized with KOH/Mops (2/0.3 M) and frozen at -20°C for subsequent determination of intracellular metabolites. Dihydroxyacetone phosphate (DHAP), Glc-6-P and fructose-6-phosphate (Fru-6-P) concentrations were measured fluorimetrically, as described [20]. Simultaneously, a second sample was also removed from the chamber and the mitochondrial and cytosolic contents were separated using the digitonin fractionation technique [24]. The cell suspension was placed in an eppendorf tube on top of a layer of an isotonic medium containing 2 mM of digitonin. After 15 sec the tube was centrifuged for 30 sec and the mitochondria were precipitated through a layer of silicon oil (Wacker AR 200) into HClO_4 (10% mass/vol.) containing 25 mM EDTA. Immediately, the supernatant was quenched with HClO_4 (5% mass/vol.). Finally, the neutralized extracts of each compartment were frozen at -20°C for subsequent determination of adenine nucleotides by high performance liquid chromatography [20].

Measurement of glucose-6-phosphatase activity

G6Pase activity was measured on microsomal fractions prepared as described [25]. Briefly, livers of overnight fasted rats were homogenized in 3 vol of 50 mM Hepes, 0.2 M sucrose, 0.5 mM EDTA, pH 7.2 in a Potter device. Microsomes were obtained by two successive centrifugations (13000 g, 20 min and 100000 g, 60 min) and kept frozen at -80°C . The microsomes were gently resuspended with a Dounce in 25 mM Hepes, 0.5 mM EDTA, pH 7.2 to reach a protein concentration of 10 mg/ml, corresponding to about 0.5 g of liver/ml. G6Pase or mannose-6-phosphatase (M6Pase) activity was measured at 30°C in a final vol of 0.2 ml containing the indicated concentrations of substrate and of silibinin (or vehicle, i.e. 0.4 % DMSO), 50 mM Hepes pH 7.2 and 0.25 mg of microsomal protein. The reaction was stopped after 20 min of incubation by the addition of 1 ml 10% TCA and the released Pi was measured colorimetrically [26]. Microsomes were permeabilized by incubation in 0.4% taurodeoxycholate for 30 min at 0°C . Microsome intactness was evaluated by measuring M6Pase activity, which was about 10-times (9.8 ± 1.4 , $n=5$; $p<0.05$) greater in permeabilized than in intact microsomes, as expected [25]. The enzyme activity was expressed as μmoles of Pi produced per min and per g of microsomal proteins and its kinetics parameters were calculated using the GOSA 2.b2 software (Bio-log, France).

Determination of oxygen consumption rate

Hepatocytes ($7.5 \text{ mg dry cells} \cdot \text{ml}^{-1}$) were incubated in a shaking water bath at 37°C in closed vials containing 2 ml Krebs-Ringer-bicarbonate-calcium buffer saturated with O_2/CO_2 (19:1)

and supplemented with 20 mM DHA. After 45 min of incubation in the presence of 100 μM silibinin or vehicle, the cell suspension was saturated again with O_2/CO_2 for one min and immediately transferred into a stirred oxygraph vessel equipped with a Clark oxygen electrode. The oxygen consumption rate was measured at 37°C and expressed in $\mu\text{mol O}_2 \cdot \text{min}^{-1} \cdot \text{g}$ dry cells $^{-1}$.

Materials

Enzymes were purchased from Roche, substrates from Merck, collagenase A, Glc-6-P, α -glycerophosphate, β -glycerophosphate, histone 2A and silibinin from Sigma. Silibinin was dissolved in a stock water-based solution containing 50% DMSO. Rhodorsil silicone oil was purchased from Rhone-Poulenc.

Statistics

The results are expressed as means $\pm$ SEM for the indicated number of separate experiments. The statistical significance of differences was calculated using Student's test for unpaired samples.

Results

Silibinin decreases both gluconeogenesis and glycolysis in hepatocytes perfused with dihydroxyacetone

In a first set of experiments we studied the effects of two different concentrations of silibinin (25 or 100 μM) on both glycolysis and gluconeogenesis rates in hepatocytes perfused with dihydroxyacetone (DHA) as exogenous substrate. Indeed, the rates of glucose (J_{glucose}) and lactate-plus-pyruvate (J_{L+P}) production strictly reflect the respective gluconeogenic and glycolytic fluxes since glycogen content is negligible in hepatocytes from 24 h-starved rats.

As shown in Figure 1A, silibinin induced a dose-dependent inhibition of gluconeogenesis from DHA, J_{Glucose} above 1.2 mM DHA exhibiting a mean reduction by 33% and 49% compared with control-vehicle in the presence of 25 and 100 μM silibinin, respectively. Since gluconeogenesis and glycolysis are branched pathways in DHA metabolism, the decrease in J_{Glucose} could be due to a concomitant increase in J_{L+P} . However, J_{L+P} was also decreased by silibinin (Figure 1B), thus leading to a drop in the overall DHA metabolism rate expressed as three-carbon equivalents (J_{DHA} , Figure 1C). This indicates that inhibition of gluconeogenesis by the flavonoid was not only related to a simple redistribution of carbon from one pathway to another. Furthermore, an increase in L/P ratio (Figure 1D), which reflects a more reduced cytosolic

Fig. 1. Effects of silibinin on metabolic pathways from DHA in perfused rat hepatocytes. Hepatocytes (200–250 mg dry cells) isolated from 24h-starved Wistar rats were perfused at 37°C. After a first steady state in absence of exogenous substrate, DHA was infused at increasing concentrations while 25 μ M (open triangles) or 100 μ M (open squares) silibinin was continuously infused in one chamber compared with a control-vehicle chamber (0.4 % DMSO, closed circles). The rates of gluconeogenesis (J_{Glucose} , A) and glycolysis ($J_{\text{L-P}}$, B) were determined. The total metabolic flux from DHA (J_{DHA} , C) was expressed as three-carbon equivalents. The effects of silibinin on cytosolic lactate-on-pyruvate ratio (D) and ATP/ADP ratio (E) and on the relationships between DHA and DHAP (F), DHAP and J_{Glucose} (G), DHAP and Fru-6-P (H), Glc-6-P and J_{Glucose} (I) were reported in the corresponding panel. The results are expressed as means $\pm$ SEM; $n=6$.

NADH/NAD⁺ redox state, and a decrease in cytosolic ATP/ADP ratio (Figure 1E) were also evidenced in presence of silibinin. This later was associated with a significant inhibition of the cellular oxygen consumption rate by silibinin (16.4 ± 1.3 versus 11.0 ± 0.5 μ mol O₂.min⁻¹.g dry cells⁻¹ for control and silibinin, respectively; $p<0.05$, $n=4$).

Silibinin inhibits glucose-6-phosphate hydrolysis

To further investigate the effect of silibinin on gluconeogenesis, the intracellular concentration of key intermediates was measured and plotted against gluconeogenic flux at each steady state, allowing the pathway to be accurately dissected. A slight drop in steady-state DHAP concentrations was evidenced in presence of silibinin (Figure 1F), suggesting that part of its effect could be due to a decrease in DHA phosphorylation.

However, a clear and dose-dependent modification of the relationship between DHAP and J_{Glucose} by silibinin was also evidenced: for a given DHAP concentration, the resultant J_{Glucose} was indeed decreased (Figure 1G), indicating that the flavonoid also exerts its inhibitory effect on gluconeogenesis downstream of DHA phosphorylation.

We next studied the effect of silibinin on the two controlling steps of the gluconeogenic pathway downstream DHAP, *i.e* the fructose and the glucose cycles. The relationships between the intracellular concentrations of DHAP and Fru-6-P were plotted, allowing the fructose-1,6-bisphosphatase (FBPase-1)/phosphofructokinase-1 (PFK-1) cycle to be investigated. According to the fact that, in our system of perfusion, i) the metabolic flux through glucokinase (GK) and PFK-1 are negligible because of the continuous wash-out of glucose and ii)

Fig. 2. Effects of silibinin on glucagon-induced stimulation of glucose-6-phosphate hydrolysis in hepatocytes perfused with DHA. Hepatocytes were perfused with increasing concentrations of DHA, as described in the legend of Figure 1, in the presence of 10^{-7} M glucagon. 100 μ M silibinin (open squares) was continuously infused in one chamber compared with a control-vehicle chamber (0.4 % DMSO, closed squares). The effects of silibinin on gluconeogenesis (J_{Glucose} , A) and glycolysis ($J_{\text{L+P}}$, B) rates and on the relationship between DHAP (C) or Glc-6-P (D) and J_{Glucose} were investigated. The results are expressed as means $\pm$ SEM; n=5.

DHAP concentration reflect the free fructose-1,6-bisphosphate level since both aldolase and triose-phosphate isomerase work at near-equilibrium [22], the relationship between DHAP and Fru-6-P is therefore expected to be only modified by changes in the metabolic flux through FBPase-1. This key controlling step was apparently not affected by silibinin (Figure 1H), indicating that its inhibitory effect on gluconeogenic pathway is located downstream of Fru-6-P. By contrast, a clear and dose-dependent modification of the relationships between both Fru-6-P (data not shown) and Glc-6-P (Figure 1I) and J_{Glucose} was evidenced, indicating a powerful inhibition of Glc-6-P hydrolysis by silibinin. Indeed, for a given Glc-6-P concentration, J_{Glucose} was decreased by about 30 and 50% compared to control-vehicle in presence of 25 and 100 μ M silibinin, respectively.

The activation of glucose-6-phosphate hydrolysis by glucagon is lowered by silibinin

As we previously reported that glucagon, besides its classical stimulatory effect on FBPase-1, stimulated gluconeogenesis from DHA by a powerful activation of

Glc-6-P hydrolysis [20], we further investigated if this specific effect could be attenuated by silibinin. In agreement with our previous work, we confirmed here that glucagon inhibited $J_{\text{L+P}}$ and increased J_{Glucose} from DHA, this latter being associated with a stimulatory effect located downstream of DHAP at the level of Glc-6-P hydrolysis (compare Figure 1 and Figure 2). Interestingly, silibinin strongly decreased the stimulation of gluconeogenesis induced by glucagon (Figure 2A) while glycolysis was only slightly affected (Figure 2B). In addition, the relationships evidenced between both DHAP (Figure 2C) and Glc-6-P (Figure 2D) and J_{Glucose} were also clearly affected, suggesting that silibinin could exerted part of its inhibitory effect on gluconeogenic pathway by lowering the glucagon-induced activation of Glc-6-P hydrolysis.

The inhibition of glucose-6-phosphate hydrolysis by silibinin was present whatever the carbohydrates sources

Since DHA is a poor physiological substrate, we further studied the effect of silibinin in hepatocytes perfused with other gluconeogenic precursors. Interest-

Fig. 3. Effects of silibinin on glucose-6-phosphate hydrolysis in hepatocytes perfused with physiological gluconeogenic precursors. Hepatocytes were perfused with increasing concentrations of lactate/pyruvate (10:1) (A), glycerol (B) or fructose (C), in presence of 100 μM silibinin (open symbols) or the vehicle (0.4 % DMSO, closed symbols). The effects of silibinin on the relationship between Glc-6-P and J_{Glucose} were plotted in the corresponding panels. The results are expressed as means $\pm$ SEM; $n=3-6$.

ingly, silibinin inhibits gluconeogenesis and Glc-6-P hydrolysis from either lactate/pyruvate (Figure 3A), glycerol (Figure 3B) or fructose (Figure 3C), although the extent of its effect varies according to the condition.

Fig. 4. Effects of silibinin on glucagon-induced glycogenolysis in perfused hepatocytes. Hepatocytes (100-120 mg dry cells) from fed Wistar rats were perfused at 37°C in the presence of 100 μM silibinin (open squares) or the vehicle (0.4 % DMSO, closed squares). After 25 min, 10^{-7} M glucagon was continuously infused to the cells. The effects of silibinin on the glucagon-induced glycogenolysis (J_{Glucose} , A) and glycolysis ($J_{\text{L+P}}$, B) rates, and on the relationship between Glc-6-P and J_{Glucose} (C) were investigated. The results are expressed as means $\pm$ SEM; $n=3$.

In addition, perfusion of hepatocytes from fed rats also reveals an inhibitory effect of silibinin on glycogenolysis rate. Indeed, the glucagon-induced stimulation of glycogenolysis was strongly reduced by the flavonoid

Fig. 5. Effect of silibinin on glucose-6-phosphatase in intact and permeabilized rat liver microsomes. In panel A, G6Pase activity was determined in intact (closed circles) and permeabilized (closed squares) rat liver microsomes (0.25 mg of microsomal protein per assay) incubated for 20 min with 10 mM Glc-6-P and increasing concentrations of the flavonoid. The release of Pi was measured colorimetrically as described in the Materials and Methods section. In panel B, experiments were performed in the presence of 250 μ M silibinin (opened circles) or vehicle (DMSO, closed circles) and increasing concentrations of Glc-6-P. The results are expressed as means $\pm$ SEM; $n=3-4$.

(Figure 4A), although the classical inhibitory effect of the hormone on glycolysis was weakly affected (Figure 4B). As evidenced with the other carbohydrate sources, the decrease in J_{Glucose} from glycogen was also clearly associated with a powerful inhibition in Glc-6-P hydrolysis (Figure 4C).

Silibinin inhibits glucose-6-phosphatase in a dose-dependent manner

Taken together, our results suggest that the inhibition of Glc-6-P hydrolysis by silibinin could be due to an effect on G6Pase. To assess this hypothesis, we measured G6Pase activity in intact and permeabilized rat liver microsomes incubated with 10 mM Glc-6-P and increasing concentrations of the flavonoid (Figure 5A). Interestingly, silibinin inhibits G6Pase activity in both intact and permeabilized microsomes by a dose-dependent manner. The extent of this effect was stronger in intact than in permeabilized microsomes (-24% versus -9% at 250 μ M, respectively), suggesting that the flavonoid mainly affects Glc-6-P transport rather than the catalytic activity of the G6Pase. Determination of G6Pase kinetics in intact microsomes (Figure 5B) indicates that silibinin does not significantly affect the K_m of the enzyme (4.8 ± 0.2 versus 5.3 ± 0.4 mM in control and silibinin, respectively; $n=3$, NS) but decreases its maximal velocity (194 ± 5 versus 146 ± 5 $\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{g proteins}^{-1}$ in control and silibinin, respectively; $n=3$, $p<0.05$).

Discussion

From the present data, silibinin appears to induce a potent inhibition of both basal and glucagon-activated glu-

coneogenesis and glycogenolysis in perfused rat hepatocytes. These newly described metabolic effects of the flavonoid could be explained in part by a decrease in Glc-6-P hydrolysis related to an inhibition of G6Pase, identifying silibinin as a potential regulator of intracellular Glc-6-P concentration.

In our experimental conditions, the glucose release (J_{Glucose}) accurately reflects the rates of gluconeogenesis from exogenous substrates or glycogenolysis from glycogen, depending on whether hepatocytes were isolated from starved or fed rats, respectively. We report here that silibinin exerts a potent inhibition of J_{Glucose} associated with a decrease in Glc-6-P hydrolysis, irrespective of the carbon sources, in both basal and glucagon-stimulated conditions. The stimulation of gluconeogenesis by glucagon was generally thought to be the sole result of its well-known inhibitory effect on pyruvate kinase, leading to carbon redistribution from glycolysis to gluconeogenesis in such a way that the total substrate flux was unaffected [22, 27, 28]. However, in agreement with our previous work [20], we confirmed that despite a powerful inhibition of $J_{\text{L+P}}$ by glucagon, the overall metabolism of DHA was increased by 20% (data not shown), reflecting an activation of gluconeogenic pathway downstream DHAP (Figure 3A-B). This effect was related to a stimulation of both flux though FBPase-1 (data not shown) and Glc-6-P hydrolysis (Figure C). Interestingly, silibinin induced a potent and specific reduction in the glucagon-induced activation of Glc-6-P hydrolysis although the flavonoid did modify neither the inhibitory effect of glucagon on $J_{\text{L+P}}$ (Figure 2B) nor the glucagon-mediated increase in flux though FBPase-1 (data not shown).

The silibinin-induced decrease in gluconeogenesis rates was however not only related to the striking effect

evidenced at the level of Glc-6-P hydrolysis. Indeed, while Glc-6-P hydrolysis was inhibited when carbons were provided from DHA, glycerol and lactate/pyruvate, the intracellular accumulation of Glc-6-P was also decreased in presence of silibinin (Figure 1 and 2), indicating other additional effect(s) on the pathway located upstream of the glucose cycle level. While the fructose cycle was apparently not affected by silibinin (Figure 1H), modifications of the ATP/ADP and/or NADH/NAD⁺ ratios could also be involved in its metabolic effect. Indeed, the decrease in cytosolic ATP/ADP, a potent regulator of gluconeogenesis, would notably reduce the phosphorylation of DHA and glycerol by glycerol kinase, thus limiting the flux of carbons into the pathway, as suggested by the decrease in both steady-state DHAP and Glc-6-P concentrations (Figure 1F-I and 3B). Similarly, the silibinin-induced increase in cytosolic NADH/NAD⁺ redox state observed with all the substrates (Figure 1E and data not shown) could also account, at least in part, for the inhibition of gluconeogenesis from glycerol. Indeed, the gluconeogenic pathway from this carbohydrate is mainly controlled by the redox-sensitive glyceraldehyde-3-phosphate dehydrogenase step. While the inhibition of oxygen consumption rate by silibinin suggests an effect of the flavonoid on the mitochondrial respiratory-chain, this issue needs to be specifically addressed in the future.

The most striking finding of this work is the potent inhibitory effect of silibinin on both Glc-6-P hydrolysis, which constitutes the common final step for glucose production from glycogenolysis and gluconeogenesis, and G6Pase activity. This latter effect evidenced in rat liver microsomes was characterized by a dose-dependent inhibition of the enzyme (Figure 5). In addition, our results indicate that silibinin inhibits G6Pase by affecting mainly Glc-6-P transport, as indirectly measured in intact microsomes, but also catalytic activity in permeabilized microsomes. We clearly showed that silibinin decreased maximal velocity of G6Pase but did not significantly modify its apparent affinity for Glc-6-P. In contrast, quercetin, another flavonoid-related compound, was reported to inhibit G6Pase by increasing its K_m for Glc-6-P without affecting the V_{max} of the enzyme [29]. This discrepancy could be related to differences in flavonoids' polarity [30], suggesting possible specificity in their interaction with both components of the G6Pase system, *i.e.* Glc-6-P translocase and catalytic subunit, depending on the type of flavonoids.

It should be stressed that another interpretation of the relationship between Glc-6-P and $J_{Glucose}$ can be provided if we take the possible existence of several Glc-6-

P pools within the cell into account [31]. Indeed, since the Glc-6-P concentration measured in our condition represents the total intracellular content of the intermediate, it could be possible that the relationship between this "averaged" Glc-6-P and $J_{Glucose}$ did not strictly reflect the carbon flux through G6Pase. For instance, some intracellular pools of Glc-6-P associated with spatially and/or metabolically separated pathways within the cell could remain inaccessible to G6Pase [32]. Thus, the channeling of the intermediate could explain the apparent sigmoidal relationship between Glc-6-P and $J_{Glucose}$, a situation mainly observed with substrates entering at the level of trioses phosphate, such as DHA, glycerol and fructose, but not with lactate/pyruvate or glycogen.

Glc-6-P is a key metabolite located at a crossroad between multiple pathways, *i.e.* gluconeogenesis, glycolysis and pentose phosphate shunt. Glc-6-P also plays a key role in the regulation of hepatic glycogen metabolism by controlling both activation state and subcellular distribution of glycogen synthase [33]. It is well recognized that hepatocytes can synthesize glycogen from gluconeogenic substrates by the so-called indirect pathway [34]. Thus, a silibinin-induced shift of carbon from glucose output to glycogen synthesis could be postulated owing to the inhibition of G6Pase by the flavonoid. However, in our condition, we did not observe any significant glycogen accumulation in either control or silibinin-perfused hepatocytes (data not shown), excluding an acute effect of the flavonoid on glycogen synthesis. Similarly, diversion of Glc-6-P from G6Pase and hepatic glucose production to the benefit of the pentose phosphate pathway (PPP) could also be hypothesized. Indeed, glucose-6-phosphate dehydrogenase (G6PdH) catalyses the first step of PPP and regulates carbon flow through this crucial pathway, which generates NADPH for both anabolic processes and protection against oxidative stress. Interestingly, inhibition of G6Pase by the antidiabetic drug metformin has been recently reported to be associated with an increased flux through G6PdH in high-fat fed rats, suggesting a mechanism of diversion of Glc-6-P from gluconeogenesis to the PPP [35]. This mechanistic explanation is consistent with the low K_m of G6PdH for Glc-6-P (~0.2 mM), a concentration close to the physiological level in the liver. Thus, any increase in Glc-6-P resulting from G6Pase inhibition would increase carbon flux through G6PdH, enhance NADPH production and regeneration of oxidized glutathione, ultimately leading to higher capacity of ROS detoxification by glutathione peroxidase. While we did not measure PPP flux in this study, it is tempting to suggest that some of the well-

known antioxidant properties of silibinin in the liver could be related to redirection of carbon from Glc-6-P toward this pathway, leading to increased NADPH generation and cellular defense against oxidative stress.

The hyperglycaemia associated with type 2 diabetes is due, in part, to an increase in hepatic glucose production resulting from both elevated circulating glucagon and uncontrolled gluconeogenesis [36]. Thus, the use of silibinin as a new plant-derived inhibitor of this pathway that also attenuates the metabolic deleterious effects of hyperglucagonemia should be interesting to normalize glycaemia in type 2 diabetics. Furthermore, since the small intestine has been recently identified as a gluconeogenic organ contributing significantly to glucose homeostasis [37], it would also be interesting to study if the high accumulation of silibinin reported in the gastrointestinal tract [38, 39] could affect G6Pase activity and gluconeogenesis in this specific tissue. Importantly, the concentrations of silibinin used in our study are only slightly higher than those found in the human liver (~ 1-5 μ M) after single oral absorption of a low therapeutic dose [39], and close to those reported in rodents [4]. Taken together, silibinin and its derivatives could be seen as safe therapeutic agents, since they exhibit particularly low toxicity. Even when given in very high concentration, they are well tolerated and are devoid of both side effects and negative drug interactions [4].

In conclusion, this study shows that *in vitro* treatment of rat hepatocytes with silibinin inhibited Glc-6-P hydrolysis and lowers glucose production from various gluconeogenic precursors and glycogen. This seems to be mediated, at least in part, by an inhibition of G6Pase by the flavonoid. While further *in vivo* studies will be necessary to address

this specific issue, we suggest that silibinin, owing to its apparent anti-gluconeogenic and anti-glycogenolytic properties, could be interesting for the management of type 2 diabetes, since reduction in hepatic glucose production constitutes a cornerstone in normalizing glycaemia in diabetics.

Abbreviations

DHA, (dihydroxyacetone); DHAP, (dihydroxyacetone phosphate); Fru-6-P, (fructose-6-phosphate); FBPase-1, (fructose-1,6-bisphosphatase); Glc-6-P, (glucose-6-phosphate); G6Pase, (glucose-6-phosphatase); G6PdH, (glucose-6-phosphate dehydrogenase); GK, (glucokinase); M6Pase, (mannose-6-phosphatase); Pi, (inorganic phosphate); PPP, (pentose phosphate pathway).

Acknowledgements

The authors are grateful to Liliane Maisin and to Francisco Alonso for technical assistance, to Drs Juan P. Bolanos, Josè M Medina and Louis Hue for stimulating discussion, and to Gitara Edward for her kind help in the revision of the manuscript. This work was supported by a grant from the Junta de Castilla y Leon (Spain), Grant Num. (SA062/03), by INSERM (Grenoble, France) and by Fundacion Renal, Salamanca, Spain. R.N. is recipient of a doctoral fellowship of AECI (Spain) and B.G. is recipient of the postdoctoral "Michel de Visscher" fellowship from the Institute of Cellular Pathology (Belgium).

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