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The actions of fisetin on glucose metabolism in the rat liver

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Fisetin is a flavonoid dietary ingredient found in the smoke tree (*Cotinus coggyria*) and in several fruits and vegetables. The effects of fisetin on glucose metabolism in the isolated perfused rat liver and some glucose-regulating enzymatic activities were investigated. Fisetin inhibited glucose, lactate, and pyruvate release from endogenous glycogen. Maximal inhibitions of glycogenolysis (49%) and glycolysis (59%) were obtained with the concentration of 200 μM. The glycogenolytic effects of glucagon and dinitrophenol were suppressed by fisetin 300 μM. No significant changes in the cellular contents of AMP, ADP, and ATP were found. Fisetin increased the cellular content of glucose 6-phosphate and inhibited the glucose 6-phosphatase activity. Gluconeogenesis from lactate and pyruvate or fructose was inhibited by fisetin 300 μM. Pyruvate carboxylation in isolated intact mitochondria was inhibited ($IC_{50} = 163.10 \pm 12.28$ μM); no such effect was observed in freeze-thawing disrupted mitochondria. It was concluded that fisetin inhibits glucose release from the livers in both fed and fasted conditions. The inhibition of pyruvate transport into the mitochondria and the reduction of the cytosolic NADH-NAD^+ potential redox could be the causes of the gluconeogenesis inhibition. Fisetin could also prevent hyperglycemia by decreasing glycogen breakdown or blocking the glycogenolytic action of hormones. Copyright © 2010 John Wiley & Sons, Ltd.

KEY WORDS — fisetin; liver perfusion; gluconeogenesis; glycogen; glucagon

INTRODUCTION

Fisetin (3,7,3́,4́-tetrahydroxyflavone) is a flavonoid dietary ingredient found in the smoke tree (*Cotinus coggyria*) but also found in fruits and vegetables such as strawberry, apple, persimmon, grape, onion, and cucumber at concentrations of 2–160 $\mu g\, g^{-1}$.[1] Flavonoids exhibit a wide variety of pharmacological properties, including antiatherosclerotic, antioxidant, anti-inflammatory, antitumor, antithrombogenic, antiosteoporotic, and antiviral effects.[2] Certain bioflavonoids, including fisetin, inhibit glycolysis and growth of tumor cells.[3]

Flavonoids have also been identified as the antidiabetic components in a number of traditional ethnic remedies.[4] Although there has been considerable scientific progress over the past few years in the unraveling of the effect and mechanism of action of flavonoids, the mechanisms whereby these compounds exert their hypoglycemic action have rarely been investigated. There are indications that the liver is implicated in this hypoglycemic action. In groups of mice whose diet was supplemented with hesperedin and naringin, increased glucokinase activity and higher glycogen content were found. Naringin also reduced the activity of hepatic glucose 6-phosphatase and phosphoenolpyruvate carboxykinase.[4] The authors have suggested that hesperedin and naringin improve hyperglycemia by regulating the activity of these hepatic enzymes involved in glycolysis and gluconeogenesis.[5] Quercetin, which is a flavonol extensively studied in relation to its potential role in diabetes, inhibits both glucose degradation and production.[6,7]

In the liver from fed rats, quercetin stimulates glycogenolysis and oxygen consumption, but inhibits glycolysis at concentrations up to 300 μM. It showed inhibitory action on the activity of hepatic glucose 6-phosphatase.[6] Also, quercetin has been demonstrated to exert a potent inhibitory effect on both glycogen phosphorylase activities a and b in isolated muscle.[8] Fisetin, which is structurally similar to quercetin, has not yet been investigated with reference to its action in the liver. However, it is known that the compound inhibits rabbit muscle phosphorylase kinase, the enzyme that activates glycogen phosphorylase.[9] Half-maximal inhibition occurs at a concentration around 50 μM.[9] This observation, in addition to the structural similarity to the active compound quercetin, leads one to the hypothesis that fisetin is equally able to affect metabolic fluxes related to carbohydrate metabolism in the liver. To investigate this possibility is exactly the purpose of the present work in which the isolated perfused rat liver was used as an experimental model.

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MATERIALS AND METHODS

Materials

The liver perfusion apparatus was built in the workshops of the University of Maringá.[10] Fisetin was purchased from Sigma Chemical Co. (St Louis, MO, USA). Enzymes and coenzymes used in the enzymatic assays were purchased from Sigma Chemical Co. (St Louis, MO, USA). Sodium [^{14}C]bicarbonate, specific activity of 58 Ci mmol^{-1}, was purchased from Amersham Pharmacia Biotech (Buckinghamshire, UK). All other chemicals were of the highest available grade.

Liver perfusion

Male Wistar rats (weighing 200–280 g) fed with a standard laboratory diet (Nuvital - Nuvilab CR-1®) were used in all experiments. All experiments were conducted in strict adherence to the guidelines of the Ethics Committee for Animal Experiments of the University of Maringá. For the surgical procedure, the animals were anesthetized by intraperitoneal injection of sodium pentobarbital (50 mg kg^{-1}). Hemoglobin-free, non-recirculating perfusion was undertaken according to the technique described by Scholz and Bücher.[11] After cannulation of the portal and cava veins, the liver was positioned in a plexiglas chamber. Flow was maintained constant by a peristaltic pump (Miniplus 3®, Gilson, France) and was adjusted to between 30 and 35 ml min^{-1}, depending on the liver weight.

The perfusion fluid was Krebs/Henseleit bicarbonate buffer (pH 7.4), saturated with a mixture of oxygen and carbon dioxide (95:5) by means of a membrane oxygenator with simultaneous temperature adjustment at 37°C. The composition of the Krebs/Henseleit bicarbonate buffer is as follows: 115 mM NaCl, 25 mM $NaHCO_3$, 5.8 mM KCl, 1.2 mM Na_2SO_4, 1.18 mM $MgCl_2$, 1.2 mM NaH_2PO_4, and 2.5 mM $CaCl_2$. Fisetin was dissolved in the perfusion fluid. Solubilization was achieved by the simultaneous addition of an equivalent amount of 1 M NaOH. When glycogen catabolism and the hepatic contents were measured, the livers of fed rats were used in the experiments. Livers from 24 h fasted rats were used for the measurement of gluconeogenesis.

Analytical

Samples of the effluent perfusion fluid were collected at 2-min intervals and analyzed for their metabolite content. Lactate and pyruvate were assayed by means of standard enzymatic procedures using L-lactate dehydrogenase.[12,13] Interference by fisetin (absorbance at 340 nm) was excluded by running blanks. The oxygen concentration in the outflowing perfusate was monitored polarographically, employing a Teflon-shielded platinum electrode adequately positioned in a plexiglas chamber at the exit of the perfusate.[14] Fisetin interferes with the glucose oxidase reaction and, for this reason, glucose was measured colorimetrically by means of an *o*-toluidine method.[15]

The hepatic contents of AMP, ADP, ATP, and glucose 6-phosphate of fed rats were measured after freeze-clamping the perfused liver with liquid nitrogen. The freeze-clamped livers were extracted with perchloric acid. The extract was neutralized with K_2CO_3 and assayed by means of standard enzymatic procedures.[16–18]

Experiments with microsomes

Microsomes from livers of 24 h fasted rats (weighing 200–280 g) were isolated by differential centrifugation according to Mihara and Sato.[19] The pellet of the last 105 000× *g* centrifugation step, containing the microsomal fraction, was suspended in cold medium (4°C) containing 150 mM KCl and 10 mM Tris (pH 7.4). The protein content was measured using the method of Lowry *et al*.[20] The standard was bovine serum albumin. The glucose 6-phosphatase activity was measured at 37°C in a medium containing 100 mM KCl, 20 mM Tris-HCl (pH 6.5), 10 mM glucose 6-phosphate and 0.2–0.3 mg protein ml^{-1}. The supernatant of the 105 000× *g* centrifugation step (high-speed centrifugation supernatant) was used for the assay of D-fructose 1,6-bisphosphatase according to the procedure described by Mendicino *et al*.[21] The reaction mixture contained 0.4–0.8 mg protein ml^{-1}, 100 mM Tris-HCl (pH 8.0), 12 mM $MgCl_2$, 1 mM D-fructose 1,6-bisphosphate, and 5 mM cysteine. Fisetin was added to the incubation medium as a solution in dimethylformamide (0.1 M). After 20 min incubation at 37°C, the reaction was interrupted by the addition of one volume of 5% trichloroacetic acid and phosphate release was measured according to the method of Fiske and Subbarow.[22]

Experiments with isolated mitochondria

Fasted rats, weighing between 200 and 280 g, were decapitated and their livers removed immediately and cut into small pieces. These fragments were suspended in a medium containing 0.2 M mannitol, 75 mM sucrose, 2.0 mM Tris-HCl (pH 7.4), 0.2 mM EGTA, 0.1 mM phenylmethylsulphonyl fluoride (PMSF), and 0.0075 mM fatty acid-free bovine serum albumin. Homogenization was carried out in the same medium by means of a Dounce homogenizer. After homogenization, the mitochondria were isolated by differential centrifugation according to Voss *et al*. using a sucrose-mannitol isolation medium, and suspended in the same medium, which was kept at 0–4°C.[23]

The pyruvate carboxylase activity of intact mitochondria was measured by a modification of the technique described by Adam and Haynes.[24] The incubation medium contained 5 mM pyruvate, 12.5 mM $MgCl_2$, 2.5 mM potassium phosphate, 120 mM KCl, 10 mM HEPES (pH 7.5), and 3 mg protein ml^{-1}. The reaction was initiated by introducing 15 mM [^{14}C]$NaHCO_3$ (0.25 μCi). After 10 min of incubation at 37°C, the reaction was arrested by the addition of 0.5 volume of 2 M perchloric acid. After expulsion of the remaining [^{14}C]$NaHCO_3$ (5 min), aliquots were taken for counting the acid stable incorporated radioactivity.

The pyruvate carboxylase of disrupted mitochondria was measured using a medium able to generate steady-state concentrations of acetyl-CoA, as originally described by Henning *et al*.[25] Rat liver mitochondria, isolated as described above, were disrupted by successive freeze and thawing procedures using liquid nitrogen. The incubation medium contained 3 mg protein ml^{-1} of disrupted mitochondria, 5 mM sodium pyruvate, 12.5 mM $MgCl_2$, 2.5 mM potassium phosphate, 0.3 M sucrose, 1 mM ethylenediamine tetraacetate (EDTA), 5 mM tris(hydroxymethyl) aminomethane (TRIS; pH 7.5), 0.5 mM lithium coenzyme A, 5 mM adenosine triphosphate, 1.1 mM acetyl phosphate, 1 unit ml^{-1} phosphotransacetylase and 1 unit ml^{-1} citrate synthase. The reaction was initiated by introducing 15 mM [^{14}C]$NaHCO_3$ (0.25 μCi). After 10 min of incubation at 37°C, the reaction was arrested by the addition of 0.5 volume of 2 M perchloric acid. After expulsion of the remaining [^{14}C]$NaHCO_3$ (5 min), aliquots were taken for counting the acid stable incorporated radioactivity.

The incorporated radioactivity in both incubations, intact and disrupted mitochondria, was expressed as μmol min^{-1} mg^{-1}. The scintillation solution for counting ^{14}C was composed of toluene/Triton x-100® (1.5/0.5), 10 g L^{-1} 1.5-diphenyloxazole plus 0.4 g L^{-1} 2,2-*p*-phenyl-bis-5-phenyleneoxazole. Protein content of all experiments was measured using the method of Lowry *et al*.[20]

Pyruvate kinase assay

Pyruvate kinase from rabbit muscle was assayed in an incubation system containing 0.1 M phosphate buffer, pH 5.3, 2.4 mM $MgSO_4$, 0.5 mM ADP, 0.5 mM phosphoenolpyruvate and 19.76 units ml^{-1} of a highly purified pyruvate kinase preparation.[26] Fisetin was added to the incubation medium as a solution in dimethylformamide (0.1 M). The reaction was initiated by the addition of phosphoenolpyruvate. After 2.5 min at 25°C, the reaction was interrupted with 2 M perchloric acid. After fisetin extraction with ethyl acetate, pyruvate production was measured in a system containing 100 mM Tris-HCl, pH 7.4, 0.25 mM NADH and lactate dehydrogenase (0.048 units ml^{-1}). The decrease in absorbance at 340 nm resulting from the extinction of NADH was measured and expressed as μmol min^{-1} mg^{-1}.

Measurement of NADH Oxidation in a cell-free system

The action of fisetin on NADH oxidation in a cell-free system was measured according to the method of Chan *et al*.[27] The reaction mixtures contained 0.1 M Tris-HCl/1.0 mM EDTA buffer (pH 7.4), fisetin (0.5–4.0 μM), H_2O_2 (25 μM), NADH (200 μM), and horseradish peroxidase (HRP) type VI-A (0.1 μM). Reactions were started by the addition of H_2O_2 (25 μM) and the oxidation of NADH was followed at 340 nm using a spectrophotometer.

Statistical analysis

The statistical significance of the differences between parameters was evaluated by means of Student's *t*-test or Newman-Keuls test. The latter was applied after submitting the data to variance analysis. The results are mentioned in the text as the *p* values; $p < 0.05$ was adopted as a criterion of significance.

RESULTS

In the first experiments, the actions of fisetin on oxygen consumption, glycogenolysis and glycolysis were investigated in the perfused liver of fed rats. Several experiments were undertaken in which fisetin was infused in the range between 50 and 300 μM during 30 min. Figure 1 illustrates the results of a representative experiment, showing the time-courses of the changes caused by 200 μM fisetin. These livers were perfused with substrate-free perfusion fluid, in an open system. Under these conditions, the livers release glucose, lactate, and pyruvate from the endogenous glycogen stores.[11] The infusion of 200 μM fisetin at 10 min of perfusion produced decreases in glucose and lactate release (62%, $p < 0.01$). At the end of the infusion, glucose release was reduced by 46% ($p < 0.001$) compared with the rates measured before the infusion of the drug. Oxygen uptake was not modified. Even when the infusion of fisetin was interrupted at 30 min, its inhibitory effects on lactate production persisted during the next 20 min. Figure 2A shows the mean results of experiments in which the effects of several concentrations of fisetin on glycogenolysis, glycolysis, and oxygen uptake were estimated at the end of the infusion period. The concentration dependence of the effects on oxygen uptake shows that oxygen uptake was not modified. Maximal inhibitions of glycogenolysis (49%, $p < 0.001$) and glycolysis (59%, $p < 0.001$) were obtained with the concentration of 200 μM. The lactate to pyruvate ratio, which is an indicator of the cytosolic NADH to NAD^+ ratio, was also clearly reduced by fisetin (Figure 2B).

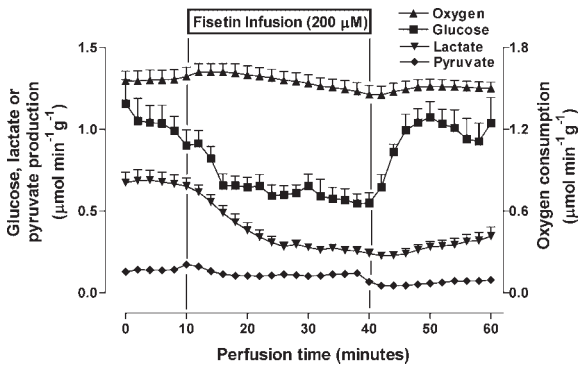

Figure 1. Time courses of the changes caused by fisetin in glycogen catabolism and oxygen uptake. Livers from fed rats were perfused as described in the Materials and methods section. Fisetin was infused at 10–40 min, as indicated by the horizontal bar. The effluent perfusate was sampled in 2-min intervals and analyzed for glucose, lactate, and pyruvate. Oxygen consumption was followed polarographically. Each data point represents the means of six liver perfusion experiments. Bars are standard errors of the mean

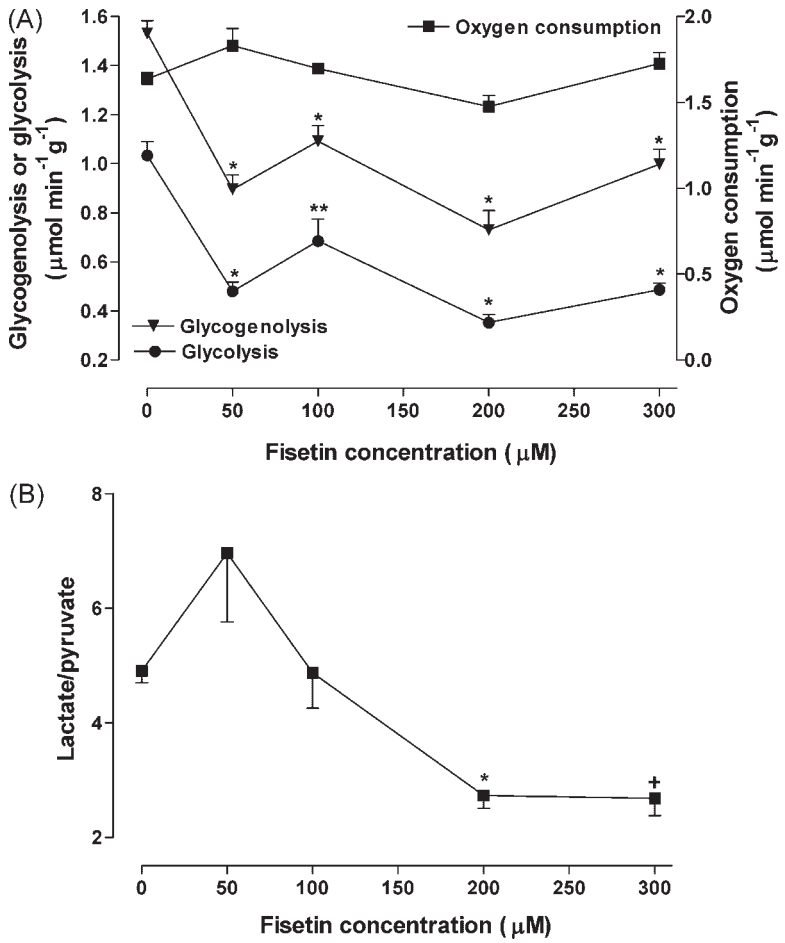

Figure 2. Concentration dependencies of the effects of fisetin on glycogen catabolism and related parameters. Livers from fed rats were perfused with Krebs/Henseleit-bicarbonate buffer (pH 7.4) with different fisetin concentrations in the range between 50 and 300 μM. The experimental protocol was the same illustrated in Figure 1. Panel A: rates of glycogenolysis and glycolysis were calculated from glucose, lactate, and pyruvate production [glycogenolysis: glucose + 1/2 (lactate + pyruvate)]; glycolysis: (lactate + pyruvate) and were expressed as glucosyl units. Panel B: lactate/pyruvate ratio. Each data point represents the means of 3–6 liver perfusion experiments. Bars are standard errors of the mean. Asterisks and crosses indicate statistical significance in comparison with the control condition as revealed by variance analysis with *post hoc* Newman-Keuls testing ($^{+}p < 0.05$, $^{**}p < 0.01$, $^{*}p < 0.001$)

Glucagon and 2,4-dinitrophenol were selected for investigating the influence of fisetin on the metabolic alterations provoked by hormones and glycogenolytic drugs. Figure 3A shows the results of the experiments in which glucagon 10 nM and fisetin 300 μM were infused in livers from fed rats. The infusion of glucagon, 10 min after initiation of the perfusate sampling, resulted in the typical effects of this hormone.[28] Both oxygen uptake and glucose release were substantially stimulated, while lactate and pyruvate production were inhibited by glucagon. The introduction of fisetin at 30 min caused a progressive reduction in both glucose production (70%, $p < 0.01$) and oxygen consumption (10%, $p < 0.05$) close to the levels seen before the infusion of the hormone. On the other hand, the lactate production was further reduced by fisetin but without statistical significance. Figure 3B illustrates the data of experiments performed with 2,4-dinitrophenol and fisetin. Fisetin was also capable to suppress the stimulatory effect of 2,4-dinitrophenol on glycogenolysis, glycolysis, and oxygen consumption because glucose release was reduced 77% ($p < 0.05$), lactate production by 89% ($p < 0.001$), and oxygen consumption by 29% ($p < 0.001$) while pyruvate production was increased 78% ($p < 0.01$).

The action of fisetin on gluconeogenesis from lactate and pyruvate was evaluated according to the protocol illustrated by Figure 4A in perfused livers from 24-h fasted rats. Under this condition, during the time period before lactate (2.0 mM) plus pyruvate (0.2 mM) infusion, glucose release was minimal due to the low endogenous levels of glycogen and gluconeogenic substrates.[29] Both gluconeogenesis and oxygen consumption increased upon addition of the exogenously supplied gluconeogenic substrates, tending to stabilize at 20 min infusion time. The introduction of 300 μM fisetin produced progressive decreases in both gluconeogenesis and oxygen consumption. At the end of the infusion, glucose production was reduced by 72% ($p < 0.001$) and oxygen consumption was inhibited 19% ($p < 0.001$) when compared with the rates measured before

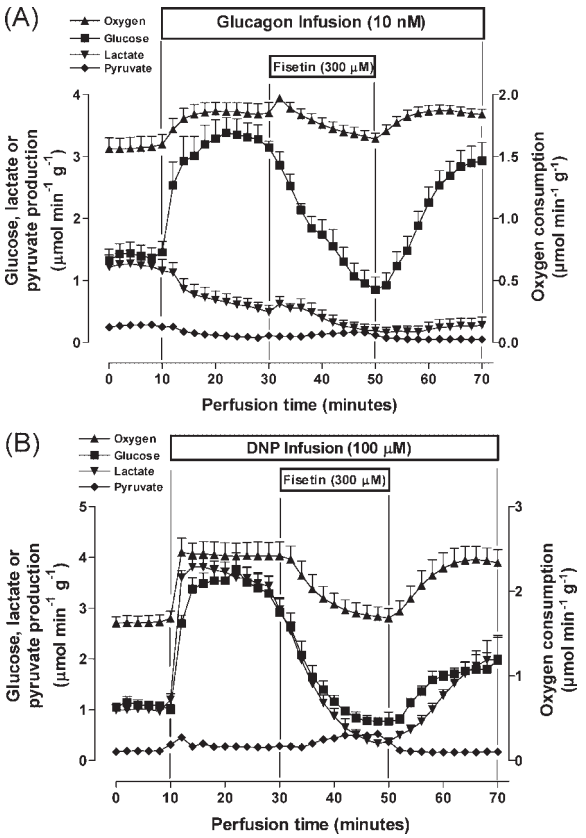

Figure 3. Effects of fisetin on metabolic fluxes in perfused livers isolated from fed rats. Livers from fed rats were perfused with Krebs/Henseleit-bicarbonate buffer (pH 7.4), as described in the Materials and methods section. Glucagon (Panel A) or 2,4-dinitrophenol (DNP) (Panel B) was infused at 10–70 min and fisetin (300 μM) at 30–50 min as indicated by the horizontal bars. The effluent perfusate was sampled in 2-min intervals and analyzed for glucose, lactate, and pyruvate. Oxygen consumption was followed polarographically. Each experimental point is the mean ± SEM of four experiments with identical protocol

the infusion of the drug. These effects were partially reversible, that is, when the infusion of the drug was interrupted at 50 min, the metabolic fluxes tended to return slowly to the rates before its infusion.

The data of Figure 4B allow an evaluation of the changes caused by several concentrations of fisetin in the range of 50–300 μM on oxygen uptake and gluconeogenesis. Gluconeogenesis was reduced in a dose-dependent manner. Inhibition of gluconeogenesis was already evident at the concentration of 50 μM and increased considerably when the concentration of the drug was raised to 200 μM (56%, $p < 0.001$).

Fructose can also be converted into glucose, but ramification of the fructose pathway at the enolase step leads also to the production of lactate and pyruvate. The action of fisetin on fructose metabolism was investigated in experiments like those shown in Figure 4A. The results summarized in Figure 5 show that glucose production from exogenously supplied fructose was inhibited 43% ($p < 0.01$) by 300 μM fisetin. Lactate and pyruvate production were also reduced; the inhibition reached 72% ($p < 0.01$) and 60% ($p < 0.01$) respectively, while oxygen consumption decreased 16% ($p < 0.01$) in the presence of fisetin.

In the search for mechanistic interpretations of the results of the liver perfusion experiments, four enzymatic activities were measured in the present work: glucose 6-phosphatase, fructose 1,6-bisphosphatase, pyruvate carboxylase, and pyruvate kinase. The results illustrated by Figure 6 reveal that the glucose 6-phosphatase activity was inhibited by fisetin in a dose-dependent manner. The mean concentration producing 50% inhibition of glucose 6-phosphatase activity was 294.40 ± 34.83 μM. Since the glucose 6-phosphatase activity was inhibited, alterations in the glucose 6-phosphate levels can also be expected.[30] In the present work, the levels of glucose 6-phosphate were measured before and after 30 min of 300 μM fisetin infusion into perfused livers isolated from fed rats as shown in Table 1. The cellular content of glucose 6-phosphate was increased 73%. On the other hand, no such effect was found on the levels of ATP, ADP, and AMP, which were measured under the same conditions.

The results of the pyruvate carboxylase measurements at various fisetin concentrations are shown in Figure 7. Although pyruvate carboxylation in isolated intact mitochondria was inhibited with a well-defined concentration dependence ($IC_{50} = 163.10 \pm 12.28$ μM), this effect was absent in freeze-thawing disrupted mitochondria. Fisetin, in the range up to 600 μM, was inactive on the fructose 1,6-bisphosphatase activity (data not shown). Fisetin was also inactive on the activity of rabbit muscle pyruvate kinase, at least in the range up to 300 μM (not shown).

Fisetin reduced the lactate to pyruvate ratio (Figure 2B). Due to the lactate dehydrogenase equilibrium this means generally a reduction in the cytosolic NADH to NAD^+ ratio, a phenomenon that could be associated with a catalytic oxidation of NADH, dependent on the action of peroxidases in the presence of H_2O_2.[31] Since both peroxidase activity and H_2O_2 are present in the liver cells, experiments were conducted in order to test this possibility using horseradish peroxidase.[31,32] The results are shown in Figure 8 in which the rate of NADH oxidation was represented against the fisetin concentration. Figure 8 clearly shows that fisetin is able to cause catalytic oxidation of NADH in the presence of H_2O_2 and peroxidase activity.

DISCUSSION

The most prominent effect of fisetin on liver metabolism that was found in the present work is its inhibitory effect on glucose output. This action results from both inhibition of gluconeogenesis and inhibition of glycogenolysis. In livers from fed rats, glycogenolysis was inhibited under basal conditions and much strongly under conditions of accelerated glycogen breakdown caused by glucagon and 2,4-dinitrophenol. In livers from fasted rats, fisetin inhibited gluconeogenesis from lactate and pyruvate and, to a lesser extent, from fructose. Mechanistic explanations for these

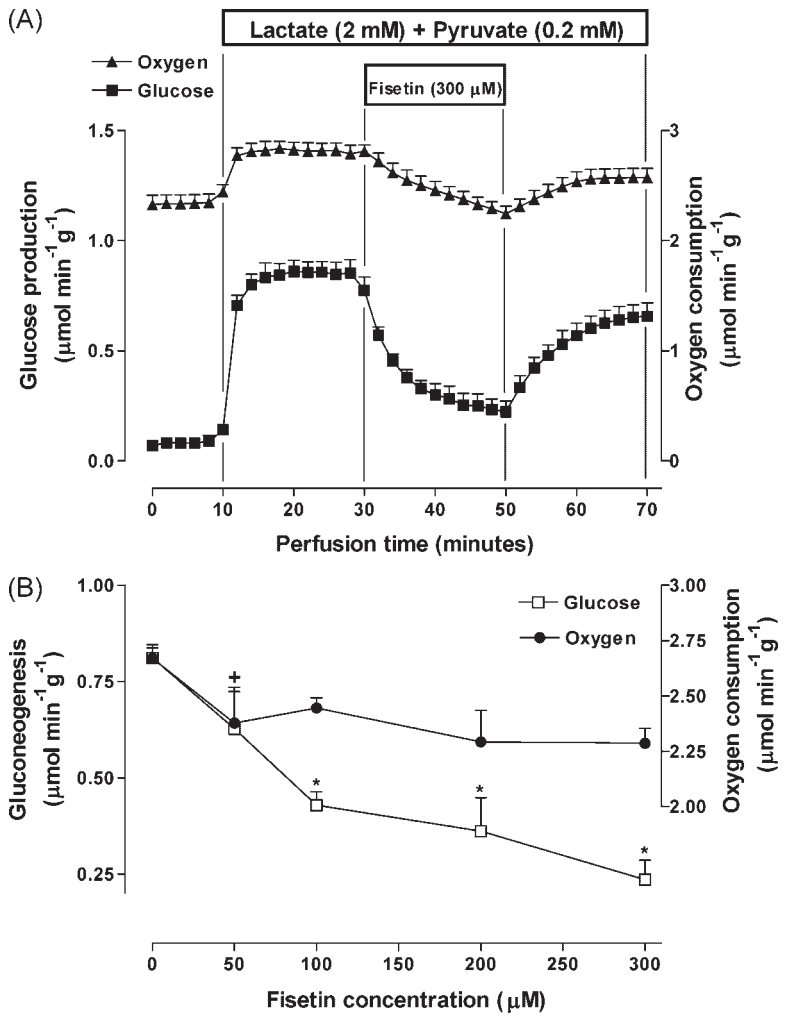

Figure 4. Effects of fisetin on metabolic fluxes in perfused livers isolated from fasted rats. Panel A: time course of the changes caused by fisetin 300 μM in glucose production and oxygen consumption. Livers from fasted rats were perfused as described in the Materials and methods section. Lactate (2 mM) and pyruvate (0.2 mM) were infused at 10–70 min and fisetin at 30–50 min as indicated by the horizontal bars. Panel B: concentration dependence of the effects of fisetin on oxygen consumption and gluconeogenesis. The experimental protocol was the same described for panel A. Each data point is the mean ± SEM of four experiments. Asterisks and crosses indicate statistical significance in comparison with the control condition as revealed by variance analysis with *post hoc* Newman-Keuls testing ($^{+}p < 0.05$, $^{*}p < 0.001$)

effects can be deduced from both literature data and from several observations done in the present work.

The main cause for gluconeogenesis diminution is evidently the inhibition of pyruvate carboxylation. This results, most probably, from an inhibition of pyruvate transport across the mitochondrial membrane, as indicated by the observation that inhibition of carboxylation is solely significant in intact mitochondria. Inhibition of pyruvate transport into the mitochondria diminishes glucose synthesis from substrates such as lactate and alanine as amply demonstrated in experiments with the classical inhibitor of pyruvate transport cyano-4-hydroxycinnamic acid[33] and also in experiments with *p*-coumaric acid.[34] Lactate must be first transformed into pyruvate before entering the gluconeogenic pathway. Inhibition of pyruvate transport limits the availability of this compound to the intramitochondrial pyruvate carboxylase which is by far the most important regulatory enzyme, as can be deduced from its flux control coefficient (between 56 and 75%) that greatly surpasses those ones of the other enzymes including phosphoenolpyruvate carboxykinase (between 0.2 and 5%).[35] Besides inhibition of pyruvate carboxylation, there are other secondary causes for gluconeogenesis inhibition by fisetin. An important one is the ability of fisetin to decrease the $NADH/NAD^+$ ratio, which is probably caused by a catalytic NADH oxidation since the liver presents several peroxidase activities[31,32] and also because H_2O_2 is always present in the hepatic cells in consequence of their metabolic activity. Gluconeogenesis from lactate or pyruvate is highly dependent on the availability of reducing equivalents especially at the glyceraldehyde 3-phosphate dehydrogenase step. The pro-oxidant effect of fisetin was also previously

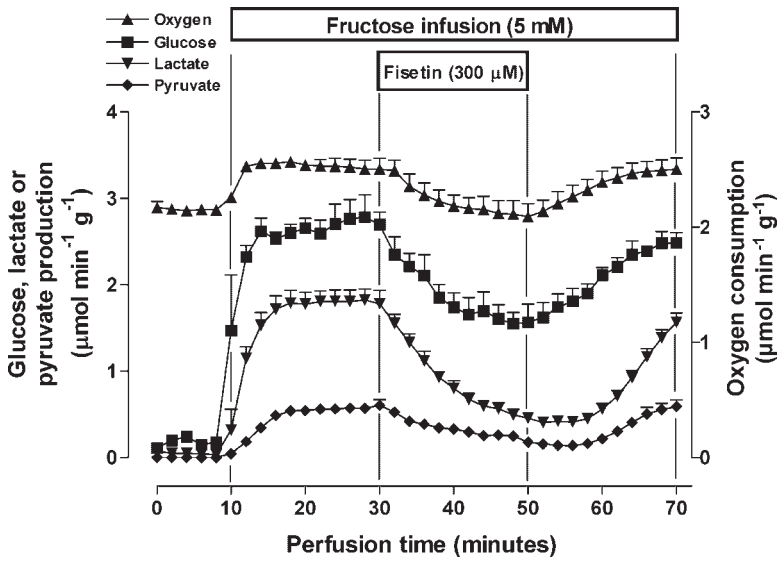

Figure 5. Effects of fisetin on fructose metabolism. Livers from fasted rats were perfused with Krebs/Henseleit-bicarbonate buffer (pH 7.4), as described in the Materials and methods section. Fructose (5 mM) was infused at 10–70 min and fisetin (300 μM) at 30–50 min as indicated by the horizontal bars. The effluent perfusate was sampled in 2-min intervals and analyzed for glucose, lactate, and pyruvate. Oxygen consumption was followed polarographically. Each experimental point is the mean ± SEM of three experiments with identical protocol

reported for quercetin, which like fisetin contains a catechol ring in its structure.[27,36]

Inhibition of pyruvate carboxylation and diminution of the availability of reducing equivalents are specific causes of the inhibitory action of fisetin on gluconeogenesis from lactate. Inhibition of glucose output due to either glycogenolysis or to transformation of fructose into glucose cannot be explained by these mechanisms. However, as already mentioned in the Introduction section, fisetin and several other flavonoids are able to inhibit phosphorylase kinase, the enzyme that activates glycogen phosphorylase.[9] Due to the continuous activation–deactivation cycle to which glycogen phosphorylase is subjected, this could be the most important mode of action of fisetin on glycogenolysis. In this respect it should be stressed that the action of fisetin on glucose output dependent on glycogenolysis is considerably more pronounced under conditions where glycogen phosphorylase is strongly stimulated, as for example in the presence of glucagon, an observation that is consistent with the idea that the compound acts via inhibition of the activation of the enzyme that catalyzes glycogen phosphorolysis. On the other hand, it cannot be excluded that fisetin also acts in a direct way on glycogen phosphorylase because this action has already been described by several flavonoids.[8,37]

Besides the specific mechanisms of action of fisetin on gluconeogenesis and glycogenolysis discussed above there are secondary mechanisms that could also affect glucose output derived from both metabolic routes. Deviation of glucosyl units for biotransformation is one such mechanism. Fisetin bears four hydroxyl groups in its structure which are prone to conjugation with glucuronic acid so that an appreciable conjugation activity can be expected. Normally

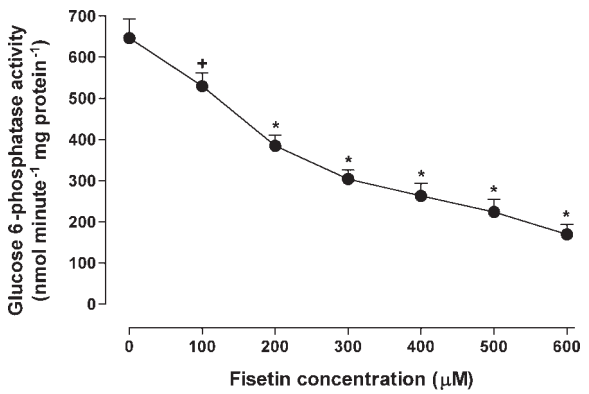

Figure 6. Action of fisetin on glucose 6-phosphatase activity. Livers from fasted rats were homogenized and subjected to differential centrifugation as described in the Materials and methods section. The microsomal fraction was used for glucose 6-phosphatase assay. Initial rates were measured at various fisetin concentrations. The data points are the means of six determinations. Bars are standard errors of the mean, $^{+}p < 0.05$, $^{*}p < 0.001$, ANOVA with Newman-Keuls test

Table 1. Adenine nucleotides and glucose 6-phosphate contents of livers from fed rats in the presence and absence of fisetin

Compound	Control ($n = 4$) μmol (g liver wet weight)$^{-1}$	Fisetin 300 μM ($n = 3$) μmol (g liver wet weight)$^{-1}$
ATP	2.089 ± 0.012	2.067 ± 0.033
ADP	0.568 ± 0.024	0.602 ± 0.017
AMP	0.176 ± 0.009	0.167 ± 0.014
Glucose 6-phosphate	0.226 ± 0.013	0.392 ± 0.003*

Isolated perfused livers from fed rats were freeze-clamped and extracted as described in the Materials and methods section. Assays were undertaken by standard enzymatic techniques. p refers to the Student's t-test.
$^{*}p < 0.001$.

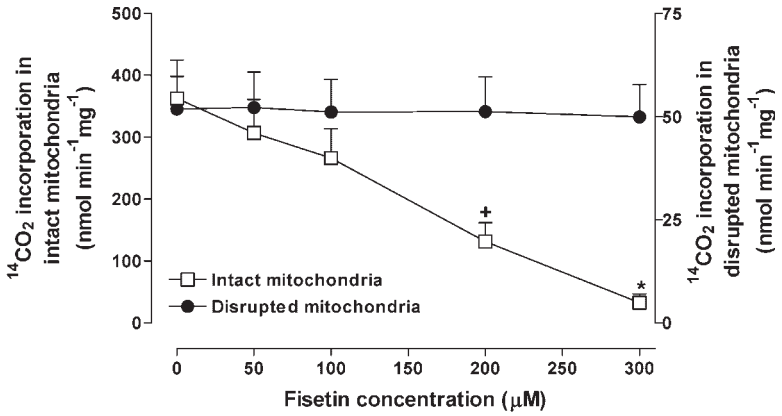

Figure 7. Action of fisetin on the activity of pyruvate carboxylase. Rat liver mitochondria were isolated as described in the Materials and methods section. Pyruvate carboxylase activity of intact mitochondria and disrupted mitochondria were measured. Each data point is the mean ± SEM of four independent experiments. $^{+}p < 0.05$, $^{*}p < 0.001$, ANOVA with Newman-Keuls test

the main source of glucuronic acid is glycogenolysis, but in the virtual absence of glycogen in livers from fasted rats, gluconeogenesis is the sole possible source.[38,39] With the data available so far, deviation of glucose 6-phosphate for glucuronidation reactions is the only explanation for the diminution of glucose output caused by fisetin when fructose was the substrate. The question if the observed inhibition of glucose 6-phosphatase by fisetin could be contributing to the inhibition of glucose output under both glycogenolytic or gluconeogenic conditions is more complex. As a terminal enzyme, it does not exert considerable control on glucose output as revealed by its very low flux control coefficient.[35] Furthermore, the K_M of this enzyme for glucose 6-phosphate is equal to 3.5 mM, about 10 times above the concentrations of this substrate.[40] Consequently, the enzyme responds almost linearly to increases in glucose 6-phosphate concentration which were indeed observed in the presence of fisetin. This increase tends to restore most of the original net flux through the enzyme. Consequently, the influence of glucose 6-phosphatase inhibition on the net glucose output is certainly of secondary importance. However, since glucose 6-phosphate is a key metabolite located between multiple pathways, including the glycogen synthesis and pentose phosphate pathways, an increase in its concentration is likely to have several consequences which could contribute to the overall effects of fisetin. In fact, glucose 6-phosphate stimulates the activity of glycogen synthase b and of glycogen synthase phosphatase, leading therefore to an increase in glycogen synthesis as well as to a decrease in glycogen degradation. It is thus possible that at the highest fisetin concentrations, the glucose 6-phosphate concentration within the cell is high enough to cause an increase in glycogen synthesis and consequently also a decrease in glycogen degradation.[41,42] Similarly, diversion of glucose 6-phosphate into the pentose phosphate pathway could also be hypothesized.[43]

The reduction of lactate and pyruvate production from endogenous glycogen is probably not the consequence of the reduced glycogen breakdown because the increased glucose 6-phosphate concentration should actually lead to an increase in the glycolytic activity. No evidence was produced in the present work which could provide an explanation for the apparently diminished glycolytic flux either from endogenous glycogen or from exogenous fructose. One can only speculate that some enzyme of the glycolytic pathway could be inhibited by fisetin. If so, inhibition of pyruvate kinase is improbable because no effect of fisetin was detected in the present work with rabbit muscle enzyme. It is important to note that no changes in the adenine nucleotides contents (ATP, ADP, etc.) were caused by fisetin in spite of the fact that inhibition of glycolysis was not accompanied by any compensatory increase in oxygen consumption. This could be indicating a reduction in the ATP demand caused by the inhibition of biosynthetic routes or of active transport systems. Clearly the causes for glycolysis inhibition by fisetin and its interrelationships with other phenomena are still demanding additional experimental efforts.

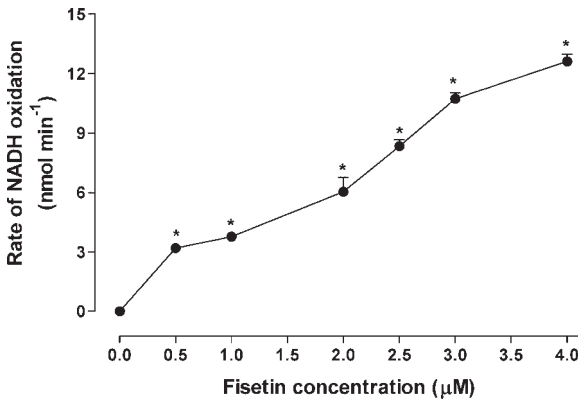

Figure 8. Dependence of the rate of peroxidase catalysed NADH oxidation on fisetin concentration. Reaction conditions: the rate of NADH oxidation was determined at 340 nm in a reaction mixture containing 0.1 M Tris-HCl/1.0 mM EDTA buffer pH 7.4, 0.1 μM horseradish peroxidase (HRP), 200 μM NADH, 25 μM H_2O_2 and different fisetin concentrations. Values represent mean ± SEM of 3–4 separate experiments. *p* refers to the ANOVA with Newman-Keuls test.$^{*}p < 0.001$

Irrespective of its mechanisms of action, the property of fisetin of reducing glucose release from the liver in both conditions, fed and fasted conditions, is remarkable. Fisetin could prevent hyperglycemia partly by decreasing glycogen breakdown or blocking the glycogenolytic action of hormones and/or by lowering hepatic gluconeogenesis. In fact, it is considered that dietary bioflavonoids may offer some protection against the early stage of diabetes mellitus,[5] since a number of studies have suggested that more than 70% of the total hepatic glucose production is due to the breakdown of glycogen in type II diabetes patients.[44] Overall, these results indicate that fisetin could be potentially useful as an antidiabetic agent, although further studies are needed to validate its therapeutic use.

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