DOI 10.1002/mnfr.200700514 Mol. Nutr. Food Res. 2009, *53*, 300–307

Research Article

# The citrus fruit flavonoid naringenin suppresses hepatic glucose production from Fao hepatoma cells

**Aparna Purushotham*, Min Tian and Martha. A. Belury**

Department of Human Nutrition, The Ohio State University, Columbus, OH, USA

Hepatic gluconeogenesis is the major source of fasting hyperglycemia. Here, we investigated the role of the citrus fruit flavonoid naringenin, in the attenuation of hepatic glucose production from hepatoma (Fao) cells. We show that naringenin, but not its glucoside naringin, suppresses hepatic glucose production. Furthermore, unlike insulin-mediated suppression of hepatic glucose production, incubation of hepatocytes with the phosphatidylinositol 3-kinase (PI3-kinase) inhibitor Ly294002 had no effect on the ability of naringenin to suppress hepatic glucose production. Further, naringenin did not increase phosphorylation of Akt at Ser473 or, Thr308, indicating this down-stream target of PI3-kinase is also not a player in naringenin-mediated suppression of hepatic glucose production. Importantly, like the dimethylbiguanide, metformin, naringenin significantly decreased cellular ATP levels without increasing cell cytotoxicity. Together, these results suggest that the aglycone, naringenin, has a role in the attenuation of hyperglycemia and may exert this effect in a manner similar to the drug, metformin.

**Keywords:** Flavonoid / Gluconeogenesis / Hepatic glucose production / Metformin / Naringenin

Received: December 17, 2007; revised: April 2, 2008; accepted: April 7, 2008

## 1 Introduction

Increased hepatic glucose production contributed by both gluconeogenesis (GNG) and glycogenolysis is the major cause for elevated fasting blood glucose levels in both type I and type II diabetes [1]. GNG alone contributes to approximately 50–60% of the endogenous glucose production [1–3] and is regulated by rate limiting enzymes such as phosphoenolpyruvate carboxy kinase (PEPCK) and glucose-6-phosphatase (G-6-Pase). The rate of transcription of these key enzymes is up-regulated by several hormones including glucocorticoids and glucagon (mediated by its second messenger cAMP). Insulin is the main hormonal suppressor of GNG and impaired insulin secretion or action together with increases in glucagon result in elevated hepatic glucose production [1, 3–5].

**Correspondence:** Dr. Martha. A. Belury, Department of Human Nutrition, The Ohio State University, 1787 Neil Ave, Columbus, OH 43210, USA
**E-mail:** belury.1@osu.edu
**Fax:** +1-614-292-8880

**Abbreviations: AMPK,** AMP-activated protein kinase; **Dex,** dexamethasone; **FBS,** fetal bovine serum; **G-6-Pase,** glucose-6-phosphatase; **GNG,** gluconeogenesis; **PEPCK,** phosphoenolpyruvate carboxy kinase; **PI3-kinase,** phosphatidylinositol 3-kinase

In addition to the usage of oral agents for reducing hyperglycemia, a great deal of attention has been given to naturally occurring compounds of plant and animal origin that may lower glucose release from the liver [6–9]. The naturally occurring compound, naringenin is a flavonone that has antiatherogenic and plasma lipid lowering effects in rodents [10–12]. A single injection of the 7-*O*-b glucoside form of naringenin reduced blood glucose, triglycerides and cholesterol in diabetic rats [13]. Naringenin exists as both glycoside–naringin and the aglycone–naringenin and is mainly derived from citrus fruits, especially grapefruit, which contains approximately 53 mg naringenin/100 g grapefruit [14]. Few studies have assessed its role in diabetes and to our knowledge the effect and mechanism of action of naringenin, the aglycone form, in lowering blood glucose is still unknown.

Recently, the glucose lowering effects of the glucoside form naringin were demonstrated in db/db mice. Feeding naringin at 0.2 g/kg diet to db/db mice significantly attenuated hyperglycemia in part by suppression of GNG along with an increase in glycogen synthesis [15]. In contrast to this novel finding, no studies have reported an effect of the

* Current address: Laboratory of Signal Transduction, National Institute of Environmental Health Sciences, NIH, 111 T. W. Alexander Drive, Research Triangle Park, NC 27709, USA.

aglycone form of naringin, naringenin on hyperglycemia. The majority of food flavonoids are bound to glycosides and in general, thought to be less bioavailable than the aglycone forms [16 – 18]. Further, a recent study using labeled naringenin reported that it was the aglycone form that readily accumulates in tissues 18 h postgavage in rats [19]. Thus, the objectives of the present study were to determine the glucose lowering effects of the aglycone naringenin *in vitro* and begin to elucidate the cellular mechanisms behind the glucose lowering effects of this citrus fruit flavonoid.

## 2 Materials and methods

### 2.1 Materials

Fao rat hepatoma cells were a gift from R. C. Kahn (Joslin Diabetes Center, Harvard, MA). Naringenin was purchased from Sigma (St. Louis, MO). DMEM, fetal bovine serum (FBS) and Trizol were purchased from Invitrogen-Gibco (Carlsbad, CA). Glucose free, phenol red free DMEM was purchased from Sigma. Naringenin, naringin, cAMP analog, 8-Ctp cAMP, synthetic glucocorticoid, dexamethasone (Dex), sodium lactate, sodium pyruvate, and insulin from porcine pancreas were purchased from Sigma. Glucose oxidase kit and trypan blue were purchased from Sigma. Naringenin and Ly294002 (Cell Signaling Technology, Beverly, MA) were solubilized in DMSO purchased from Sigma. Metformin was purchased from Sigma. PEPCK antibody was a gift from Dr. Colleen Croniger (Case Western Reserve University, Cleveland, OH).

### 2.2 Glucose output experiments

Glucose output experiments were performed using a modified protocol from Kahn *et al*. [4]. Fao cells (passages 11 – 17) were grown in low glucose DMEM containing 10% FBS and plated on six-well plates at $1.6 \times 10^6$ cells *per* well. Experiments were performed once cells reached confluence. At confluence, medium was changed to 0.1% FBS containing low glucose DMEM and incubated overnight to equilibrate cells. Next morning, cells were washed and incubated in gluconeogenic medium (GNG) – glucose free, phenol red free DMEM containing 20 mM Na lactate and 2 mM Na pyruvate as indicated. Cells were stimulated with 8-Ctp-cAMP (100 μM) plus Dex (500 nM) to induce GNG and treated with naringenin and naringin as indicated. Medium was collected at the end of the incubation period and analyzed for glucose secreted into the medium using glucose oxidase kit. Cells were washed $2\times$ with ice cold PBS, harvested and lysed in $10\times$ Tris w/v buffer containing 20 mM trizma base, 1% triton-X100, 50 mM NaCl, 250 mM sucrose, 50 mM NaF, 5 mM $Na_4P_2O_7 \cdot 10\,H_2O$ and protease inhibitors. Cell lysates were centrifuged at $12\,000 \times g$ for 10 min at 4°C and supernatants were collected and stored at −80°C. Protein concentration was determined using the bicinchoninic assay protein determination kit (Pierce Biochemicals, Rockford, IL). Glucose production was normalized to protein content *per* sample. In addition to naringenin, insulin and metformin (dimethylbiguanide) are two agents that suppress hepatic glucose production and were used in all experiments as comparative controls. Ly294002 a specific phosphatidylinositol 3-kinase (PI3-kinase) inhibitor was used as indicated. DMSO was used as vehicle control in all experiments containing naringenin at a final concentration of 0.1% v/v. All experiments were performed at least three times and representative data are shown.

### 2.3 Real time RT-PCR

Fao cells were incubated in GNG medium for 6 h with test compounds and RNA was isolated using Trizol reagent. Extracted RNA was diluted in RNase-free water and quantified by spectrophotometry. The first transcripts were reverse transcribed using reverse transcriptase (Applied Biosystems, Foster City, CA) and cDNA was amplified using real-time PCR with FAM labeled TaqMan gene expression assays (Applied Biosystems). In short, 10 ng of the reverse transcription reaction was amplified in a total reaction volume of 25 μL using predesigned and validated primers for liver PEPCK and G-6-Pase using universal cycling conditions. Target gene expression was normalized to VIC labeled 18 s, which was used as an endogenous control and amplified in the same reaction as the target gene.

### 2.4 Western blotting

Western blot analysis was performed for detecting proteins that modulate glucose metabolism. Cells were incubated in GNG medium containing cAMP plus Dex with insulin, naringenin, and metformin as indicated for 6 h. Cells were harvested and protein extracts were prepared as described earlier in Section 2. Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes (BioRad, Hercules, CA). Membranes were blocked overnight at 4°C with primary antibodies to detect p-Akt (1:1000) and PEPCK (1:5000) followed by incubation with HRP linked secondary antibody (1:2500). Polyclonal antibodies to p-Akt (Ser473 and Thr308) were purchased from Cell Signaling Technology. Total Akt (1:1000) and β-actin (1:1000) antibodies were used to normalize values. Proteins were detected using super signal chemiluminescence system (Pierce Biochemicals). Blots were quantified using densitometry (Kodak Imaging System, Rochester, NY).

### 2.5 ATP content

Cellular ATP content was determined using cell titer-Glo luminescent cell viability assay (Promega, Madison, WI) in a reaction catalyzed by recombinant firefly luciferase.

 

Mono-oxygenation of luciferin is catalyzed by luciferase in the presence of $Mg2^{+}$, ATP, and oxygen generating luminescence proportional to the amount of ATP present in the cells. Fao cells were cultured as described previously and plated in 96-well plates in low glucose DMEM. Cells were stimulated with cAMP plus Dex and treated with DMSO, naringenin (0 – 100 μM), and metformin (1 mM) in GNG medium for 6 h. Relative luminescence was normalized to total protein content/sample.

### 2.6 Cell viability

Fao cells were plated at $1.6 \times 10^6$ cells *per* well on six-well plates in low glucose DMEM and at confluence; cells were incubated with treatments in GNG medium for 6 h. At the end of the incubation period, cells were trypsinized and cell viability was determined using the method of trypan-blue exclusion. In addition, cell membrane integrity was determined using CytoTox-One homogeneous membrane integrity assay (Promega). Membrane integrity was assessed by measuring the amount of lactate dehydrogenase released from cells that were plated and cultured in low glucose DMEM containing 10% FBS in 96 well plates. Cells were incubated with different treatments as indicated in 0.1% FBS containing low glucose DMEM. Relative fluorescence proportional to the number of lysed cells was used as a measure of cell membrane integrity.

### 2.7 Statistical analysis

All data are presented as mean ± SEM. Data were analyzed using MINITAB (version 14, State College, PA). Data were analyzed by one-way ANOVA. *Post-hoc* analysis was performed using Tukey's test. Differences were considered significant at $p < 0.05$.

## 3 Results

### 3.1 Naringenin inhibits hepatic glucose production in a dose dependent manner

Glucose production was significantly increased in cells stimulated with 8-Ctp-cAMP plus Dex compared to unstimulated cells. Insulin significantly decreased glucose production in 8-Ctp-cAMP plus Dex-stimulated cells. Treatment with naringenin also significantly decreased glucose production in a dose dependent manner compared to DMSO-vehicle treatment (Fig. 1A). The glucose suppressive effect of naringenin was greatest with the 100 μM dose and interestingly, was significantly lower than unstimulated cells. Metformin, a known inhibitor of GNG also significantly decreased glucose production at 1 mM concentration.

In order to determine the glucose suppressive effects of naringenin over time, Fao cells were incubated in GNG medium with cAMP plus Dex as indicated (Fig. 1B). The glucose lowering effect of naringenin was earlier (at 2 h) than both insulin and metformin persisted up to 12 h. In contrast to the marked glucose lowering effect of naringenin, treatment with the glucoside naringin at comparable doses had no effect on hepatic glucose output in Fao cells (Fig. 1C).

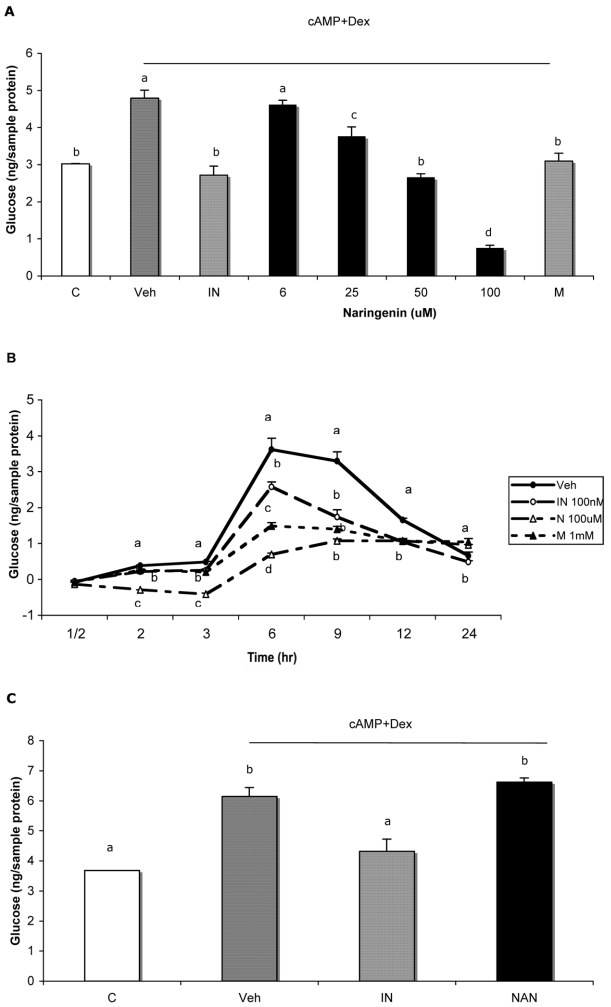


**Figure 1.** Regulation of hepatic glucose production by naringenin. As shown in (A) glucose secreted into the medium was measured after stimulating cells with cAMP plus Dex in GNG medium for 6 h. Stimulated cells were treated with Veh (DMSO), naringenin (N; 6-100 μM), insulin (IN; 100 nM), and metformin (M; 1 mM). Unstimulated cells are represented as (C) in all experiments. (B) Effect of Veh (DMSO), naringenin (N; 100 μM), insulin (IN; 100 nM), and metformin (M; 1 mM) on hepatic glucose production over time. Superscripts represent significant differences between treatments within time points. (C) Glucose suppressive effect of glucoside naringin (NAN; 100 μM) after 6 h in GNG medium. Values represent mean ± SEM. Superscripts represent significant differences between treatments, $p < 0.05$. Representative data from 3 to 4 different experiments are shown.

 

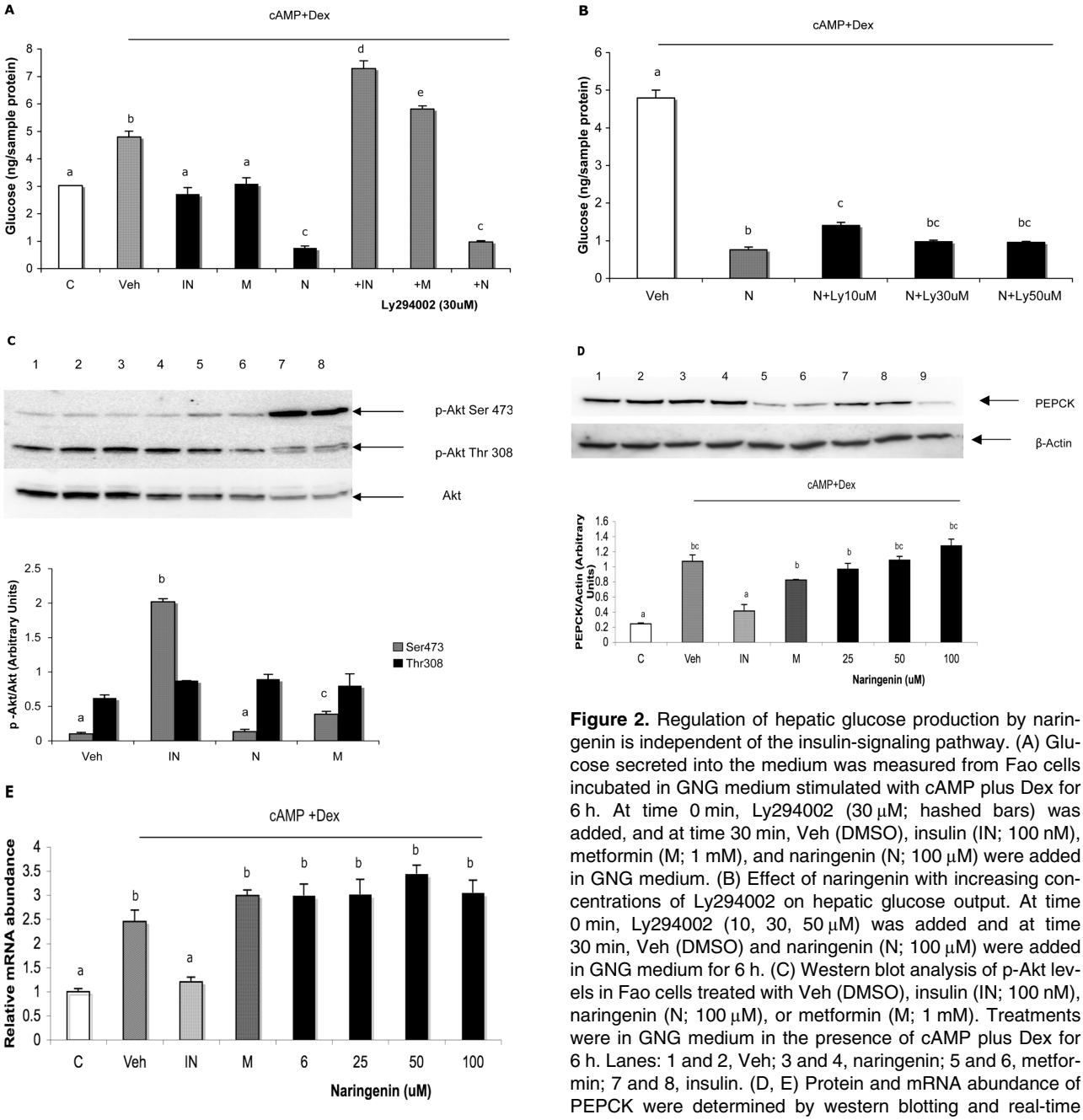


**Figure 2.** Regulation of hepatic glucose production by naringenin is independent of the insulin-signaling pathway. (A) Glucose secreted into the medium was measured from Fao cells incubated in GNG medium stimulated with cAMP plus Dex for 6 h. At time 0 min, Ly294002 (30 μM; hashed bars) was added, and at time 30 min, Veh (DMSO), insulin (IN; 100 nM), metformin (M; 1 mM), and naringenin (N; 100 μM) were added in GNG medium. (B) Effect of naringenin with increasing concentrations of Ly294002 on hepatic glucose output. At time 0 min, Ly294002 (10, 30, 50 μM) was added and at time 30 min, Veh (DMSO) and naringenin (N; 100 μM) were added in GNG medium for 6 h. (C) Western blot analysis of p-Akt levels in Fao cells treated with Veh (DMSO), insulin (IN; 100 nM), naringenin (N; 100 μM), or metformin (M; 1 mM). Treatments were in GNG medium in the presence of cAMP plus Dex for 6 h. Lanes: 1 and 2, Veh; 3 and 4, naringenin; 5 and 6, metformin; 7 and 8, insulin. (D, E) Protein and mRNA abundance of PEPCK were determined by western blotting and real-time PCR respectively from Fao cells treated with Veh (DMSO), naringenin (N; 6–100 μM), insulin (IN; 100 nM), and metformin (M; 1 mM) for 6 h in GNG medium in the presence of cAMP plus Dex. Lanes: 1 and 2, DMSO; 3 and 4, naringenin (100 μM); 5 and 6, insulin; 7 and 8, metformin; 9, unstimulated cells (C). Values represent mean ± SEM. Superscripts represent significant differences between treatments, $p < 0.05$.

### 3.2 Inhibition of hepatic glucose production by naringenin is independent of PI3-kinase signaling

Insulin regulates hepatic glucose production in a PI3-kinase dependent manner. To test whether the suppressive effect of naringenin, like insulin, was dependent on activation of PI3-kinase, cells were treated with a specific PI3-kinase inhibitor, Ly294002 (30 μM) at time 0 min, and at time 30 min, DMSO, naringenin, insulin, or metformin were added to the GNG medium and incubated for 6 h. Ly294002 not only completely blocked insulin and metformin's suppressive effects on glucose output, but also, increased glucose production in Fao cells treated with insulin and metformin. In contrast, naringenin's suppression of

glucose production was unaffected by Ly294002 (Figs. 2A and B).

Activation of Akt/protein kinase B (PKB), a downstream signaling molecule in the PI3-kinase pathway occurs by phosphorylation at least two sites Ser473 and Thr308. Activated Akt is a key regulator of glucose metabolism. Therefore, we sought to determine if naringenin, like insulin, mediates its glucose suppressive effects *via* Akt signaling. Fao cells were incubated in GNG medium with insulin, naringenin, or metformin. Activation of Akt was determined by measuring phosphorylation at Ser473 and Thr308 residues of Akt protein after 6 h. While insulin increased phosphorylation of the Ser473 residue on Akt significantly compared to DMSO treated cells, naringenin did not increase Akt phosphorylation at this residue. Phosphorylation of Akt at Thr308 was not altered significantly by any treatments (Fig. 2C). Similar patterns of induction of phospho Ser473-Akt by insulin were observed after 3 h and 9 h. Yet, naringenin had no significant effects on Akt phosphorylation at these other time points as well (data not shown).

### 3.3 Measurements of PEPCK mRNA and protein

PEPCK is one of the key enzymes regulating GNG during fasting. In order to determine the involvement of PEPCK in the suppression of glucose production from Fao cells, we measured mRNA and protein levels of PEPCK. Treatment with cAMP plus Dex significantly increased mRNA and protein levels of PEPCK compared to unstimulated cells. Insulin significantly suppressed PEPCK transcript and protein levels in cAMP plus Dex-stimulated cells. In contrast, naringenin and metformin did not alter either mRNA or protein levels of PEPCK under similar conditions (Figs. 2D and E). In addition to the data shown here, PEPCK transcript levels were measured at several time points (4 – 24 h). No significant differences were observed with naringenin treatment (data not shown).

### 3.4 Substrate does not alter suppression of hepatic glucose production by naringenin

Uptake of lactate into the mitochondria is the rate-limiting step for its conversion to oxaloacetate and subsequently, the export of lactate into the cytosol for conversion to glucose. Therefore, we tested the possibility that inhibition of hepatic glucose production by naringenin could be altered by decreased lactate uptake into mitochondria. Glucose production from Fao cells was measured using sodium lactate, oxaloacetate, or glycerol plus sodium pyruvate. Because oxaloacetate and glycerol bypass the rate limiting step of uptake into mitochondria, we tested the possibility of a blunting effect of suppression of glucose output by naringenin which would indicate the mitochondrial uptake step of lactate is required. Naringenin significantly suppressed glucose production compared to DMSO treated cells regardless of the substrate used (Figs. 3A – C). Similarly, suppression of hepatic glucose production by insulin or metformin was unaltered by substrate (oxaloacetate or glycerol) in Fao cells (data not shown).

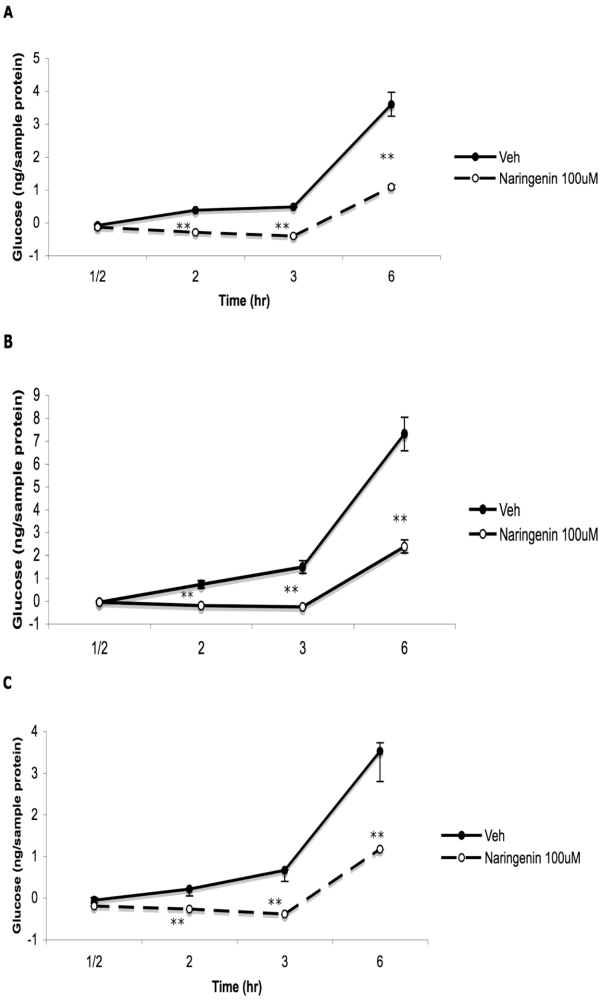


**Figure 3.** Effect of gluconeogenic substrates on hepatic glucose production by naringenin. Glucose secreted into the medium was measured from Fao cells incubated with (A) 20 mM Na lactate ± 2 mM Na pyruvate; (B) 20 mM oxaloacetate ± 2 mM Na pyruvate; and (C) 20 mM glycerol ± 2 mM Na pyruvate in the presence of cAMP plus Dex. Values represent mean ± SEM. ** represent significant differences between treatments at each time point, $p < 0.05$.

### 3.5 Cellular ATP determination and cell viability

Recent evidence suggests that metformin inhibits GNG by decreasing the mitochondrial ATP production by inhibiting complex I of the respiratory chain [20]. In order to test the possibility that naringenin exerts its effects on glucose production by a similar mechanism, cellular ATP concentrations were measured using a luciferase based assay. Compared to DMSO treated cells, naringenin and metformin

 

significantly decreased ATP concentration (Fig. 4A). These changes were observed in the absence of changes in viability determined by trypan-blue exclusion (Fig. 4B) and LDH assay (Fig. 4C). Protein concentration was also unchanged with naringenin treatment (data not shown).

## 4 Discussion

In the present study, we show for the first time that the aglycone naringenin is a potent suppressor of hepatic glucose production most likely involving some mechanisms that are similar to those recognized for the dimethylbiguanide drug, metformin. Fao cells, derived from the H4IIE hepatoma line have been extensively used to study hepatic glucose production *in vitro* as these cells produce glucose from non-glucose substrates and can survive in glucose free medium [4]. Therefore, we chose this *in vitro* model to determine the effects of naringenin on hepatic glucose production in the present study.

The effects of insulin and to a lesser extent, metformin on the inhibition of hepatic glucose output have been well characterized. Here, we have identified another potent inhibitor of glucose output, naringenin, which robustly inhibited glucose production from Fao cells. It has been previously demonstrated that Fao cells maintained in low glucose medium (5.5 mM) lack substantial amounts of glycogen [5]. The lack of substantial amounts of glycogen in Fao cells makes this a good model to predict for glucose released into media to be primarily from *de novo* synthesis from nonhexose precursors such as lactate and pyruvate.

A previous study using db/db mice showed similar glucose lowering effects using the glucoside naringin [12]. In contrast to these findings in mice, we show here that naringenin, but not the glucoside naringin, significantly suppressed hepatic glucose production when used at comparable doses. Naringin is the glucoside form of the flavonoid and undergoes cleavage of the glucose moiety by bacteria in the gut before it is absorbed [21]. Therefore, it is possible that the effects of naringin observed by Jung *et al*. [15, 22] were in fact, due to naringenin, which is possibly the active form.

We did not observe significant effects of insulin, cAMP or naringenin on G-6-Pase expression (data not shown). These results are in agreement with findings by Argaud *et al*. [23], who reported that Fao cells express very low levels of both glucokinase and G-6-Pase. Metformin has been shown to decrease G-6-Pase activity along with increases in glycogen stores with minimal effects on PEPCK gene expression in livers of rats [24, 25]. Similar to G-6-Pase, there was no evidence that metformin and naringenin alter PEPCK mRNA expression in the present study using Fao cells. It is possible that like metformin, naringenin may exert its glucose lowering effects by inhibiting G-6-Pase expression and needs to be examined in future studies using *in vivo* models and primary hepatocytes that have sufficient amounts of G-6-Pase for detection.

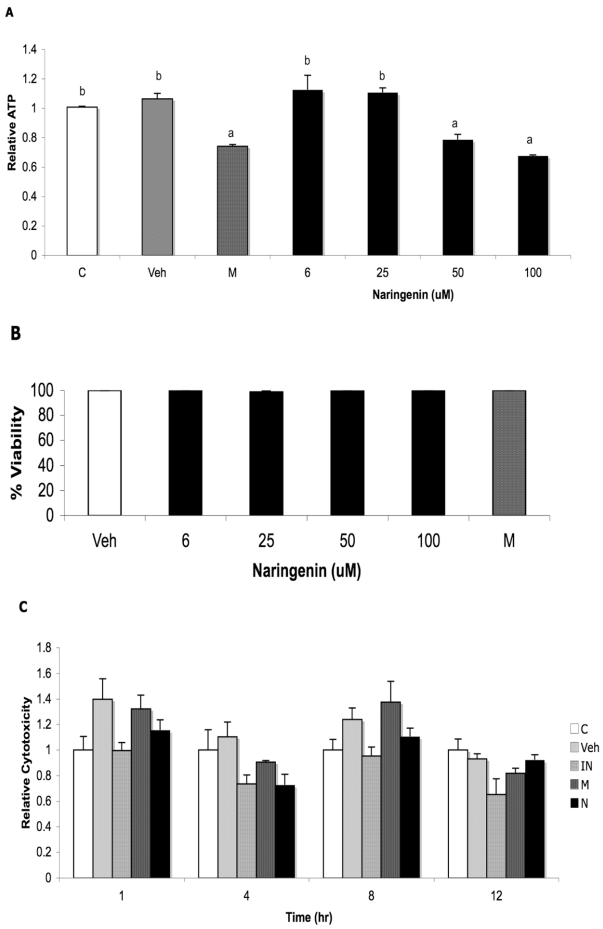


**Figure 4.** Effect of naringenin on cellular ATP concentrations and cell viability. (A) Cellular ATP concentration was determined after 6 h of incubation with Veh (DMSO), naringenin (N; 6 – 100 μM), and metformin (M; 1 mM) in GNG medium in the presence of cAMP plus Dex. (B) Cell viability was determined by trypan-blue exclusion method after 6 h of incubation in GNG medium in the presence of cAMP plus Dex. Cells were treated with naringenin (N; 6 – 100 μM) or metformin (M; 1 mM). Viability is shown relative to Veh (DMSO) treatment. (C) Cells were incubated with Veh (DMSO), insulin (IN; 100 nM); metformin (M; 1 mM); or naringenin (N; 100 μM) in low glucose DMEM without phenol red for indicated time points. Cytotoxicity was determined using LDH assay and values relative to unstimulated cells (C) are shown. Values represent mean ± SEM. Superscripts represent significant differences between treatments, $p < 0.05$.

In the present study, while specific PI3-kinase inhibitor Ly294002 significantly attenuated both insulin and metformin's ability to suppress hepatic glucose production, blocking PI3-kinase activity had no effect on the ability of naringenin to suppress hepatic glucose production. Furthermore, in the present study, naringenin did not significantly increase phospho-Akt at the Ser473 or Thr308 residues.

 

Because activation of Akt by PI3-kinase is an important signaling event responsible for the metabolic actions of insulin, these data suggest that naringenin, unlike insulin, mediates its effects on hepatic glucose production in a non-PI3-kinase dependent manner.

Metformin is a widely used compound for the attenuation of hyperglycemia mainly by inhibiting hepatic glucose production. Postulated mechanisms of action to suppress GNG include inhibition of lactate uptake into the mitochondria, inhibition of rate limiting enzyme activities (mainly G-6-Pase) and increased flux through pyruvate kinase secondary to decreased cellular ATP content [20, 24, 26 – 29]. In the present study, we explored some of these possible mechanisms with naringenin. To test the possibility that naringenin decreased hepatic glucose production by blocking mitochondrial lactate uptake, we used oxaloacetate and glycerol, as these substrates are intermediates in the gluconeogenic pathway that bypass the rate-limited requirement for uptake into mitochondria. However, in the present study, naringenin's ability to suppress hepatic glucose production was not altered when oxaloacetate and glycerol were used as substrates compared to comparable concentrations of lactate. These data suggest that hepatic glucose production suppression by naringenin is not dependent on substrate uptake in Fao cells. Further, in contrast with studies using isolated rat hepatocytes showing decreased lactate uptake with metformin [26], in Fao cells we did not see differences in glucose output when lactate, oxaloacetate or glycerol were used.

It is worth noting that the magnitude of suppression of glucose production by naringenin was significantly greater than metformin up to 6 h. These findings suggest that naringenin may alter early events in the activation of gluconeogenic pathway. The suppression of glucose output by naringenin also occurs at lower doses (50 – 100 μM) than metformin (effective only at much higher doses such as 1 – 2 mM) for immediate effects [28].

There is substantial evidence suggesting that metformin suppresses hepatic glucose production by activating AMP-activated protein kinase (AMPK) [24, 28]. In the present study however, the effect of incubating Fao cells with either naringenin or metformin on phosphorylation of AMPK was very minor (data not shown). Recent evidence suggests that metformin exerts its antidiabetic effects by inhibition of the mitochondrial respiratory chain complex I [20, 27, 30]. Inhibition of the respiratory chain complex results in decreased cellular ATP synthesis thereby increasing cytosolic ADP: ATP ratio and consequently, a decrease in pyruvate carboxylase function along with an increase in the pyruvate kinase flux, which in turn is associated with decreased GNG [24, 29]. In the present study, we show a similar effect of both metformin and naringenin on decreasing cellular ATP concentrations. In this study, we tested doses of naringenin that are comparable to previously reported concentrations by Borradaile *et al*. [11] in HepG2 cells. At these concentrations, naringenin did not have any toxic effects on Fao cells as determined by trypan-blue exclusion and cell cytotoxicity.

In conclusion, we have identified the aglycone naringenin to be the active form and a very potent suppressor of glucose output using an *in vitro* cell culture based model and the mechanism of action of the aglycone naringenin appears to be similar but not identical to mechanisms of the biguanide, metformin. Future work using primary hepatocytes and *in vivo* studies will help to clarify the importance and mechanisms of suppression of hepatic glucose production by naringenin as this could lead to improved therapeutic targets for the effective management of diabetes and associated hyperglycemia.

*We would like to thank the Carol S. Kennedy Professorship, The Edith M. Slabaugh Scholarship Fund and the Vivian Endowment Fund and Fix/Abbott Research Award for supporting this work. We thank Dr. Angie Wendel, Dr. Li-Fen Liu and Michelle Asp for their help with experiments.*

*The authors have declared no conflict of interest.*

## 5 References

[1] Bays, H., Mandarino, L., DeFronzo, R. A., Role of the adipocyte, free fatty acids, and ectopic fat in pathogenesis of type 2 diabetes mellitus: Peroxisomal proliferator-activated receptor agonists provide a rational therapeutic approach, *J. Clin. Endocrinol. Metab*. 2004, *89*, 463 – 478.

[2] Hundal, R. S., Krssak, M., Dufour, S., Laurent, D., *et al*., Mechanism by which metformin reduces glucose production in type 2 diabetes, *Diabetes* 2000, *49*, 2063 – 2069.

[3] Song, S., Andrikopoulos, S., Filippis, C., Thorburn, A. W., *et al*., Mechanism of fat-induced hepatic gluconeogenesis: Effect of metformin, *Am. J. Physiol.* 2001, *281*, E275 – E282.

[4] Kahn, C. R., Lauris, V., Koch, S., Crettaz, M., Granner, D. K., Acute and chronic regulation of phosphoenolpyruvate carboxykinase mRNA by insulin and glucose, *Mol. Endocrinol.* 1989, *3*, 840 – 845.

[5] Shao, J., Qiao, L., Janssen, R. C., Pagliassotti, M., Friedman, J. E., Chronic hyperglycemia enhances PEPCK gene expression and hepatocellular glucose production via elevated liver activating protein/liver inhibitory protein ratio, *Diabetes* 2005, *54*, 976 – 984.

[6] Collins, Q. F., Liu, H. Y., Pi, J., Liu, Z., *et al.*, Epigallocatechin-3-gallate (EGCG), a green tea polyphenol, suppresses hepatic gluconeogenesis through 5′-AMP-activated protein kinase, *J. Biol. Chem.* 2007, *282*, 30143 – 30149.

[7] Wolfram, S., Raederstorff, D., Preller, M., Wang, Y., *et al*., Epigallocatechin gallate supplementation alleviates diabetes in rodents, *J. Nutr.* 2006, *136*, 2512 – 2518.

[8] Govorko, D., Logendra, S., Wang, Y., Esposito, D., *et al*., Polyphenolic compounds from *Artemisia dracunculus* L. inhibit PEPCK gene expression and gluconeogenesis in an H4IIE hepatoma cell line, *Am. J. Physiol.* 2007, *293*, E1503 – E1510.

[9] Witters, L. A., The blooming of the French lilac, *J. Clin. Investig.* 2001, *108*, 1105 – 1107.

 

[10] Bok, S. H., Lee, S. H., Park, Y. B., Bae, K. H., *et al*., Plasma and hepatic cholesterol and hepatic activities of 3-hydroxy-3-methyl-glutaryl-CoA reductase and acyl CoA: Cholesterol transferase are lower in rats fed citrus peel extract or a mixture of citrus bioflavonoids, *J. Nutr.* 1999, *129*, 1182 – 1185.

[11] Borradaile, N. M., de Dreu, L. E., Huff, M. W., Inhibition of net HepG2 cell apolipoprotein B secretion by the citrus flavonoid naringenin involves activation of phosphatidylinositol 3-kinase, independent of insulin receptor substrate-1 phosphorylation, *Diabetes* 2003, *52*, 2554 – 2561.

[12] Borradaile, N. M., de Dreu, L. E., Barrett, P. H., Behrsin, C. D., Huff, M. W., Hepatocyte apoB-containing lipoprotein secretion is decreased by the grapefruit flavonoid, naringenin, via inhibition of MTP-mediated microsomal triglyceride accumulation, *Biochemistry* 2003, *42*, 1283 – 1291.

[13] Choi, J. S., Yokozawa, T., Oura, H., Improvement of hyperglycemia and hyperlipemia in streptozotocin-diabetic rats by a methanolic extract of Prunus davidiana stems and its main component, prunin, *Planta Med.* 1991, *57*, 208 – 211.

[14] USDA database for the flavonoid content of selected fruits, release 2.1 (2007); http://www.ars.usda.gov/Services/docs.htm?docid=6231.

[15] Jung, U. J., Lee, M. K., Jeong, K. S., Choi, M. S., The hypoglycemic effects of hesperidin and naringin are partly mediated by hepatic glucose-regulating enzymes in C57BL/KsJ-db/db mice, *J. Nutr.* 2004, *134*, 2499 – 2503.

[16] Rechner, A. R., Kuhnle, G., Bremner, P., Hubbard, G. P., *et al*., The metabolic fate of dietary polyphenols in humans, *Free. Radic. Biol. Med*. 2002, *33*, 220 – 235.

[17] Felgines, C., Texier, O., Morand, C., Manach, C., *et al*., Bioavailability of the flavanone naringenin and its glycosides in rats, *Am. J. Physiol. Gastrointest. Liver Physiol.* 2000, *279*, G1148 – 1154.

[18] Ross, J. A., Kasum, C. M., Dietary flavonoids: Bioavailability, metabolic effects, and safety, *Annu. Rev. Nutr.* 2002, *22*, 19 – 34.

[19] El Mohsen, M. A., Marks, J., Kuhnle, G., Rice-Evans, C., *et al*., The differential tissue distribution of the citrus flavanone naringenin following gastric instillation, *Free. Radic. Res*. 2004, *38*, 1329 – 1340.

[20] Owen, M. R., Doran, E., Halestrap, A. P., Evidence that metformin exerts its anti-diabetic effects through inhibition of complex 1 of the mitochondrial respiratory chain, *Biochem. J.* 2000, *348*, 607 – 614.

[21] Choudhury, R., Chowrimootoo, G., Srai, K., Debnam, E., Rice-Evans, C. A., Interactions of the flavonoid naringenin in the gastrointestinal tract and the influence of glycosylation, *Biochem. Biophys. Res. Commun.* 1999, *265*, 410 – 415.

[22] Jung, U. J., Lee, M. K., Park, Y. B., Kang, M. A., Choi, M. S., Effect of citrus flavonoids on lipid metabolism and glucose-regulating enzyme mRNA levels in type-2 diabetic mice, *Int. J. Biochem. Cell. Biol*. 2006, *38*, 1134 – 1145.

[23] Argaud, D., Kirby, T. L., Newgard, C. B., Lange, A. J., Stimulation of glucose-6-phosphatase gene expression by glucose and fructose-2,6-bisphosphate, *J. Biol. Chem.* 1997, *272*, 12854 – 12861.

[24] Cleasby, M. E., Dzamko, N., Hegarty, B. D., Cooney, G. J., *et al*., Metformin prevents the development of acute lipid-induced insulin resistance in the rat through altered hepatic signaling mechanisms, *Diabetes* 2004, *53*, 3258 – 3266.

[25] Mithieux, G., Guignot, L., Bordet, J. C., Wiernsperger, N., Intrahepatic mechanisms underlying the effect of metformin in decreasing basal glucose production in rats fed a high-fat diet, *Diabetes* 2002, *51*, 139 – 143.

[26] Radziuk, J., Zhang, Z., Wiernsperger, N., Pye, S., Effects of metformin on lactate uptake and gluconeogenesis in the perfused rat liver, *Diabetes* 1997, *46*, 1406 – 1413.

[27] El-Mir, M. Y., Nogueira, V., Fontaine, E., Averet, N., *et al*., Dimethylbiguanide inhibits cell respiration via an indirect effect targeted on the respiratory chain complex I, *J. Biol. Chem.* 2000, *275*, 223 – 228.

[28] Zhou, G., Myers, R., Li, Y., Chen, Y. *et al*., Role of AMP-activated protein kinase in mechanism of metformin action, *J. Clin. Investig.* 2001, *108*, 1167 – 1174.

[29] Argaud, D., Roth, H., Wiernsperger, N., Leverve, X. M., Metformin decreases gluconeogenesis by enhancing the pyruvate kinase flux in isolated rat hepatocytes, *Eur. J. Biochem.* 1993, *213*, 1341 – 1348.

[30] Hardie, D. G., Neither LKB1 nor AMPK are the direct targets of metformin, *Gastroenterology* 2006, *131*, 973; author reply 974 – 975.

 