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Cyanidin-3-O-β-glucoside and protocatechuic acid activate AMPK/mTOR/S6K pathway and improve glucose homeostasis in mice

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ABSTRACT

AMP-activated protein kinase (AMPK) activation is an established treatment for diabetes. Here the effects of anthocyanin extract from blood orange juice, i.e. cyanidin-3-O-β-glucoside (C3G) and its secondary metabolite protocatechuic acid (PCA), on AMPK signalling in murine hepatic cell line and in the liver of C57BL/6 mice were investigated. Results showed for the first time that C3G and PCA activate AMPK and suppress its downstream kinase mTOR/S6K both *in vitro* and *in vivo* systems. Then, C3G and particularly PCA increased expression of glut 1 and glut 4 in the liver and improved glucose tolerance in normal and obese mice. Finally, the consumption of blood orange juice was observed to increase insulin sensitivity in mice. These findings indicate that C3G and PCA are natural AMPK activators and dietary consumption of food sources of C3G and PCA improves glucose homeostasis. Mainly, blood orange juice has beneficial effect for type 2 diabetes.

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1. Introduction

Blood orange cultivars are characterised by the presence in the flesh and in the rind, of red pigments belonging to the anthocyanin class. Juice from blood oranges is an important dietary source of C3G and PCA (Vitaglione et al., 2007) and has been shown to have beneficial effects on obesity and glucose homeostasis (Grosso et al., 2013).

The prevalence of type 2 diabetes (T2D) is increasing, reaching 2% of people worldwide (Day, 2001). Currently available therapies for diabetes include insulin and various oral antidiabetic agents such as sulphonylureas, biguanides, α-glucosidase inhibitors induce various adverse effects (Saxena & Vikram, 2004). Most of the available drugs for T2D are derived directly or indirectly from plants. It may be mentioned in this connection that the discovery of widely used hypoglycaemic drug, metformin came from the traditional approach of using

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Abbreviations: C3G, cyanidin-3-glucoside (PubChem CID: 23724730); EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol tetraacetic acid; FBS, foetal bovine serum; GTT, glucose tolerance test; HFD, high fat diet; ITT, insulin tolerance test; PCA, protocatechuic acid (PubChem CID:611); T2D, type 2 diabetes; WB, Western blot; SD, standard diet

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Galega officinalis (Bailey & Day, 2004) The candidate target capable of mediating the beneficial effects of metformin is AMP activated kinase (AMPK) (Towler & Hardie, 2007) AMPK is a heterotrimeric protein consisting of a catalytic α and regulatory β and γ subunits (Hardie, 2014) that are activated by two distinct signals: a Ca2+-dependent pathway mediated by CaMKKb and an AMP-dependent pathway mediated by LKB1 (Sanders, Grondin, Hegarty, Snowden, & Carling, 2007).

AMPK has wider role in metabolic regulation (Hardie & Carling, 1997) this includes muscle glucose uptake, fatty acid oxidation (Hayashi, Hirshman, Kurth, Winder, & Goodyear, 1998; Merrill, Kurth, Hardie, & Winder, 1997; Winder & Hardie, 1999), expression of cAMP stimulated gluconeogenic genes such as PEPCK and G6Pase (Goodyear, 2000) and glucose stimulated genes associated with hepatic lipogenesis, including fatty acid synthase (FAS), Spot-14, and pyruvate kinase (Lochhead, Salt, Walker, Hardie, & Sutherland, 2000). AMPK also stimulates translocation of the glucose transporters Glut 1 and Glut 4, to the plasma membrane to enhance glucose entry into the cell (Foretz, Carling, Guichard, Ferre, & Foufelle, 1998). Notably, these effects of AMPK on glucose uptake can occur independently of insulin action, which is beneficial in insulin-resistant individuals. At molecular levels, complex relationship exists between AMPK and insulin signalling pathways.

An important modulator of insulin action is the mammalian target of rapamycin (mTOR), a member of the phosphoinositide kinase-related family that possesses exclusively protein kinase activity. mTOR functions in a mitogenic pathway downstream of PI3K and is activated by insulin and other mitogens in the presence of sufficient nutrients such as amino acids and glucose (Yang & Guan, 2007). Activated mTOR regulates protein synthesis via phosphorylation of its targets, such as activation of S6 kinase 1 (p70S6K1) and inhibition of the initiation factor 4E binding protein (4E-BP1). In addition, mTOR and S6K1 have been shown to induce serine/threonine phosphorylation of IRS1 to attenuate signal flow to downstream effectors, and thus play a role in insulin resistance. In contrast, when cells sense a shortage of nutrients, for instance, reduced cellular levels of glucose, or other stresses that deplete intracellular ATP, mTOR is inhibited and protein synthesis slows down, allowing ATP to be used for processes more critical to survival. This event is largely controlled by AMPK (Ginion et al., 2011). In fact, many studies have shown that the activation of AMPK leads to an inhibition of mTOR/S6K1 (Hardie, 2008).

Anthocyanins, including cyanidin, are naturally occurring polyphenolic compounds in the plant foods and widely distributed in fruits, vegetables and pigmented cereals and have been reported to have important effect on mammalian metabolism. They inhibit fat accumulation (Titta et al., 2010; Tsuda, Horio, Uchida, & Aoki, 2003) and are involved in glucose-induced insulin release from pancreatic β-cells (Jayaprakasam, Vareed, Olson, & Nair, 2005). Cornus fruits (cherry), a rich source of anthocyanins, have been reported to have anti-diabetic activity (Yamahara et al., 1981) since years. It was also reported that several compounds present in grape skin or whole grapes are capable of enhancing insulin secretion as well as selectively inhibiting COX-2 enzymes (Zhang et al., 2004). Moreover, it was demonstrated that purified dietary C3G reduces the blood glucose level and improves insulin sensitivity in type 2 diabetic mice (Sasaki et al., 2007). Notably, PCA (3,4-dihydroxybenzoic acid) derived from the B ring of C3G was detected as an abundant anthocyanin metabolite (Tsuda, Horio, & Osawa, 1999), thought to be formed during anthocyanin metabolic processing by enteric bacteria or in chemical reactions (Kay, Kroon, & Cassidy, 2009) it has also been found in humans (Azzini, Vitaglione, Intorre, & Napolitano, 2010; Kahle et al., 2006; Vitaglione et al., 2007). The administration *in vivo* of PCA resulted in reduced gene expression of lipogenic enzymes in the liver, thus leading to a decreased hepatic lipid accumulation (Liu, Lin, Wang, Mong, & Yin, 2010) and exerted insulin like effects by up regulating PPARγ activity (Scazzocchio et al., 2011). Finally, anthocyanin-rich bilberry extract was shown to improve glucose tolerance in type 2 diabetic patients (Hoggard et al., 2013) and to increase insulin sensitivity in mice via activation of AMP-activated protein kinase (Takikawa, Inoue, Horio, & Tsuda, 2010). However, whether anthocyanins act directly on AMPK is still unclear.

Here we present the results of cell culture and mouse models experiments aimed to validate the effect of C3G and PCA from blood orange juice on AMPK/mTOR/S6K pathway and on glucose homeostasis.

2. Methods and materials

2.1. *Reagents*

C3G-enriched extract was obtained from the juice of the blood orange variety *Moro*. Oranges were collected at maturity at Palazzelli (Siracusa, Italy) in the experimental farm of the Research Center for Citric culture and Mediterranean Crops (Acireale, Italy). Fruits were immediately stored at +4 °C and few days later squeezed. C3G was obtained by extracting the pre-filtered *Moro* juice (total anthocyanin content 74.60 mg/l as determined by pH differential method) (Rapisarda, Fanella, & Maccarone, 2000) with methanol containing 1% HCl for 1 h at + 4 °C and centrifuged at 20,000 *g* for 20 min at 4 °C. Methanol was removed by rota vapour at +30 °C in vacuum. The dried sample was dissolved in water and passed through a RP-18 g (Waters, Milford, MA, USA) pre-equilibrated with methanol. After washing with acidified H_2O to remove sugars and phenolic acids, the adsorbed anthocyanins were eluted with methanol containing 0.1% of HCl. Finally, after removal of the methanol through rota vapour, the dried sample was dissolved in water and stored at –20 °C until use.

Cyanidin concentration (C3G ≥ 95% was determined by pH differential method. Briefly, an aliquot of extract (10 μl) was diluted to 1 ml with a 0.2 M potassium chloride pH 1.0 solution and a second aliquot (10 μl) was diluted to 1 ml with a 0.4 M sodium acetate pH 4.5 solution. After 15 min incubation at room temperature the absorbance of the solutions was measured at 510 nm. Concentration was calculated by the equation: $C_{mg/l} = (Abs_{510}\ pH\ 1.0 - Abs_{510}\ pH\ 4.5) \times 484.82_{\ C3G\ MW} \times 1000/24{,}825_{\ C3G\ molar\ absorbidity} \times$ Dilution Factor.

Purified PCA (HPLC ≥ 90%) was purchased from Extrasynthese (Lyon, France), Cat. no. 6050 and dissolved in water.

Compound C was purchased from Sigma-Aldrich (St. Louis, MO, USA). Antibodies against AMPK, pAMPK (Thr-172), mTOR, p-mTOR (Ser2448), p70S6K, p-p70S6K (Thr-389) were from Cell Signaling Technology (Danvers, MA, USA). Anti-actin and

anti-vinculin antibodies were from Sigma-Aldrich. Glut 1 and Glut 4 antibodies were from Santa Cruz Biotechnologies (Santa Cruz, CA, USA).

2.2. Cell culture

BNL CL.2 (ATCC Cat # TIB-73) murine hepatocytes and Hela cells were obtained from the American Type Culture Collection and cultured in Dulbecco's modified Eagle's media (DMEM) containing 10% foetal bovine serum (FBS), 1% antibiotics (penicillin and streptomycin), 1% glutamin at 37 °C in a humidified atmosphere of 5% CO_2. After reaching 80% confluence, prior to treatment cells were serum starved for 12 hours and treated for 6 hours with C3G and PCA at a concentration of 0.5 mM and control received only vehicle (saline). Metformin at a concentration of 0.5 mM was used as a positive control. Experimental conditions were determined upon preliminary trials, incubating the cells with different concentrations of C3G and PCA (10–500 mM) for different times (1–24 hours).

2.3. Protein isolation and Western blot (WB)

After the treatment, cells were harvested by trypsinisation, centrifuged and lysed in 0.1 ml of lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% NP-40, 50 mM NaF, 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM Ethylene glycol-bis(2-aminoethyl ether)-N,N,N,N-tetraacetic acid (EGTA), 1 mM Na_3VO_4, 1 mM dithiothreitol, 1% Triton X-100, 10% glycerol and protease inhibitor cocktail). The tissues were homogenised with 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 50 mM NaF, 5 mM sodium pyrophosphate, 10% glycerol, 1% Triton X-100, 1 mM sodium metavanadate, 0.5 mM phenylmethylsulphonyl fluoride, 1 mM dithiothreitol, and a protease inhibitor cocktail (P8340, Sigma-Aldrich SRL, Milan, Italy) at 40 °C. The homogenate was centrifuged at 10,000 *g* for 15 min at +4 °C. The protein concentration of the obtained supernatant was determined using a Protein Assay system (Bio-Rad, Hercules, CA, USA) with bovine serum employed as a standard. Aliquots of the supernatant were treated with Laemmli sample buffer for 5 min at +100 °C.

Total protein was quantified by Bradford's method. Equal amount of protein (25 μg) was separated by electrophoresis in 6 or 10% SDS-polyacrylamide gel, transferred to polyvinylidene fluoride membranes and probed with specific antibodies. Blots were treated with appropriate secondary antibodies conjugated with horseradish peroxidase followed by enhanced chemiluminescence detection (Amersham Bio-sciences, Buckinghamshire, UK). Equal loading of proteins was verified by immunoblotting with anti-vinculin antibody. Densitometric analysis was performed with Image J software.

2.4. AMPK activity assay

AMPK kinase activity was determined using the CycLex AMPK Kinase Assay Kit (CycLex Co., Ltd., Nagano, Japan) according to the manufacturer's instructions. Briefly, recombinant active human AMPK (α1β1γ1; Sigma-Aldrich) was incubated with the indicated concentrations (10 μM, 100 μM and 500 μM) of AMP, metformin, C3G and PCA for 30 min at 30 °C in a pre-coated plate with a substrate peptide corresponding to mouse insulin receptor substrate-1 (IRS-1). The phosphorylation of Ser-789 in IRS-1 was determined using an anti-mouse phospho-Ser-789 IRS-1 monoclonal antibody and peroxidase-coupled anti-mouse IgG antibody. Conversion of the chromogenic substrate tetra-methylbenzidine was quantitated by absorbance measurement at 450 nm.

2.5. Measurement of intracellular ATP levels

BNL cells were plated on 96 well plates at a density of 20,000 cells/well in DMEM containing 10% FBS were treated for 6 hours with vehicle (saline) or C3G, PCA and metformin at 50 μM concentration. Total cellular ATP was measured according to the supplier's instructions (ATP Assay Kit; Promega, Madison, WI, USA).

2.6. Isolation and polarographic analysis of mitochondria

Liver mitochondria were prepared from C57Bl/6 mice scarified by cervical dislocation. Isolated liver was finely chopped, homogenised in a buffer containing 0.25M sucrose, 10 mM Tris-HCl pH 7.4, 0.1 mM EGTA and centrifuged at 650 *g* for 10 min at 20C. The supernatant was centrifuged again at 7000 *g* for 10 min and the pellet containing mitochondria was resuspended in ice cold buffer containing 0.25 M sucrose, 10 mM Tris-HCl pH 7.4, 20 μM EGTA.

Respiration rate by isolated mitochondria was obtained with oxygraph (Hansatech-Instruments LTD). Briefly, mitochondria were transferred to the electrode chamber in 0.25 M sucrose, 10 mM Tris-HCl pH 7.4, 20 μM EGTA, 200 μM ADP, 4 mM/l NaH_2PO_4, 4 mM/l $MgCl_2$, 5 mM/l Succinate and attained to a steady rate of oxygen consumption. Thereafter, compounds (10–500 μM) were added and oxygen consumption was recorded.

2.7. Animal studies

In all, 8 weeks old (approximately 18–20 g of body weight) C57/Bl6 male mice were purchased from Harlan laboratories. Mice were housed in an air-conditioned room (temperature 210 °C, relative humidity 60 ± 10%) with a white–red light cycle (lights on from 0830 to 2030 h). Group housing (four animals per cage) was chosen to improve animal's welfare. Home cages were Plexiglas boxes (42 × 27 × 14 cm) with sawdust as bedding. All animals had access to food (standard diet, SD, 2018S Teklad Global 18% Protein Rodent Diet, Harlan Laboratories or high fat diet, HFD, D12492, with 60% kcal from fat, Research Diets, New Brunswick, NJ, USA) and water ad libitum, unless otherwise stated.

Experiments have been done in accordance with institutional guidelines and the Italian law, which enforces EU directives regarding the protection of animals used for experimental and other scientific purpose.

To evaluate the acute *in vivo* effect of C3G and PCA on AMPK/mTOR/S6K signalling pathway, mice were randomly divided into four groups (n = 3). C3G, PCA (1 mg/g body weight in 300 μl water) was orally administered by gavage to 2 hours fasted animals. This dose and time were chosen based on pilot experiments. Control animals received water. Metformin was used as positive control. One hour later, the animals were sacrificed and liver were isolated immediately and frozen in liquid nitrogen for protein extraction and WB analysis.

2.8. Glucose and insulin tolerance tests and lipid profile

The effect of C3G and PCA on peripheral blood glucose levels upon glucose or insulin intraperitoneal injection, glucose tolerance test (GTT) and insulin tolerance test (ITT) respectively, was performed on 2 hours – fasted animals (n = 6/group) treated orally with C3G, PCA (1 g/kg body weight) or vehicle (water). At 1 hour after treatment, the animals received glucose (2 g/kg body weight) or insulin (0.5 Unit/kg; Sigma-Aldrich). The blood glucose levels were measured immediately before and 20, 40, 60, 80 min via blood drops obtained by clipping the tail of the mice using an ACCU-CHEK Advantage II Glucose Monitor (Roche Diagnostics, Indianapolis, IN, USA). The effect of 2 months dietary administration of blood (*Moro* cultivar) or blond (*Naveline* cultivar) orange juice as drinking water to mice fed high fat diet on ITT was performed on 6 hours fasted animals (n = 6/group) upon injection of 0.4 U/kg insulin.

Blood triglycerides, cholesterol and free fatty acid levels were measured using Reflotron Plus system from Roche.

2.9. Data analysis

Paired Student t test was used for the comparison of results from biochemical assays between treatments or groups and a p value 0.05 or 0.01 are considered statistically significant. One-way ANOVA analysis was used for GTT and ITT data comparisons among treatments. The results are presented as means ± SEM and a p value 0.05 or 0.01 are considered statistically significant.

3. Results

3.1. C3G and PCA activate AMPK and down regulate mTOR/S6K pathway in BNL cells

To investigate the effect of C3G and PCA on AMPK activation in cell culture systems, the phosphorylation at Thr-172 of AMPK was determined in mouse embryonic hepatocytes (BNL CL.2 cells). The cells were serum starved overnight and then treated with 0.5 mM of C3G-enriched extract (C3G) or PCA for 6 hours respectively. Metformin at 0.5 mM concentration was used as a positive control. WB analysis with antibodies that recognise total AMPKα subunit or specifically the phosphorylated Thr-172 α subunit revealed that the phosphorylation of AMPKα at Thr 172 was significantly increased 1.5 ± 0.4 folds by C3G and 1.7 ± 0.4 folds by PCA (Fig. 1 A) when compared to control (cells treated with vehicle only).

Several evidences indicate that AMPK inhibits the mTOR/S6K pathway (Hardie, 2008) To this end, we determined whether C3G and PCA affects mTOR-S6K pathway by analysing the levels of phosphorylation of Ser-2448 of mTOR and Thr-389 of S6K in BNL cells by WB analysis using specific antibodies. Treating BNL cells with 0.5 mM C3G and PCA caused a significant reduction in the phosphorylated serine residue of mTOR (Fig. 1B) by 1.5 ± 0.02 fold in case of C3G and 2.2 ± 0.19 fold in case of PCA. The decrease in phosphorylation of mTOR was accompanied by decrease in the phosphorylation levels of the kinase p70S6K (Fig. 1C) which was about 2 ± 0.13 fold in C3G treated cells and of 7 ± 0.02 fold in PCA treated cells.

All together these findings indicate that C3G and PCA, in the mM range, activate AMPK and suppress the TOR-S6K pathway in BNL cells.

3.2. C3G and PCA activate AMPK in liver

To validate the effects of C3G and PCA on AMPK/mTOR/S6K pathway *in vivo*, 8 weeks old C57Bl/6 male mice were randomly divided into four groups (n = 3), treated orally by gavage with 1 mg/g of body weight C3G, PCA, metformin or water. One hour later, livers were collected and their protein extracts were analysed by WB using antibodies against total AMPK and pAMPK (Thr 172). Immunoblot analysis showed a significant increase in phosphorylation of AMPK in the liver, 2.1 ± 0.4 fold higher in C3G and 2.6 ± 0.5 fold higher in PCA when compared to control mice treated with water (Fig. 2A). Notably, the phosphorylation of AMPK induced by C3G and PCA was higher than the known AMPK activator metformin.

Then, we examined the effects of C3G and PCA treatment of mice on mTOR/S6K pathway in liver tissue. Liver tissue extracts obtained from mice treated with C3G and PCA were used as a source to investigate the mTOR/S6K activation state as well. As shown in the WB picture of Fig. 2B mTOR signal was variable among individual animals and treatments with metformin, C3G and PCA did not result in significant effects on the phosphorylation of Ser 2448 of liver mTOR (Fig. 2B). However, the phosphorylation level of 70S6K on Thr-389, which correlates with the kinase activity, was decreased significantly by the treatments. As shown in Fig. 2C the reduction of phosphoThr 389 S6K was about 4.5 fold in C3G (C3G *vs.* control $p < 0.01$) treated and almost complete inhibition in PCA treated tissues compared to untreated control (PCA *vs.* control $p < 0.001$). The metformin treated animals show only a modest decrease in the phosphorylation level of S6K compared to the control (Fig. 2C).

These findings indicate that acute treatment of C3G and PCA significantly activate AMPK and down regulate S6K in mouse livers.

3.3. C3G and PCA activate AMPK upstream kinase

To elucidate the mechanism responsible for the AMPK activation by C3G and PCA, we first checked whether C3G and PCA activate purified AMPK directly by an *in vitro* assay. The incubation of recombinant human AMPK (α1β1γ1) with different concentrations 10 μM, 100 μM and 500 μM of C3G, PCA and metformin failed to activate AMPK revealed that C3G and PCA are not direct activators of AMPK (Fig. 3A).

We also treated simultaneously BNL cells with anthocyanins and compound C, which selectively inhibits AMPK activity by acting as a competitive inhibitor of ATP binding to the catalytic α subunit of AMPK. The effect of compound C on AMPK activity in BNL cells were determined by WB analysis after treating with compound C at non toxic concentration 20 μM along with 500 μM of C3G and PCA for 6 hours. Interestingly compound C has no effect on phosphorylation of AMPK in both C3G and PCA treated cells (Fig. 3B). Then, the treatment with C3G and PCA failed to promote phosphorylation of AMPK in LKB1 deficient Hela cells as revealed by WB analysis (Fig. 3C)

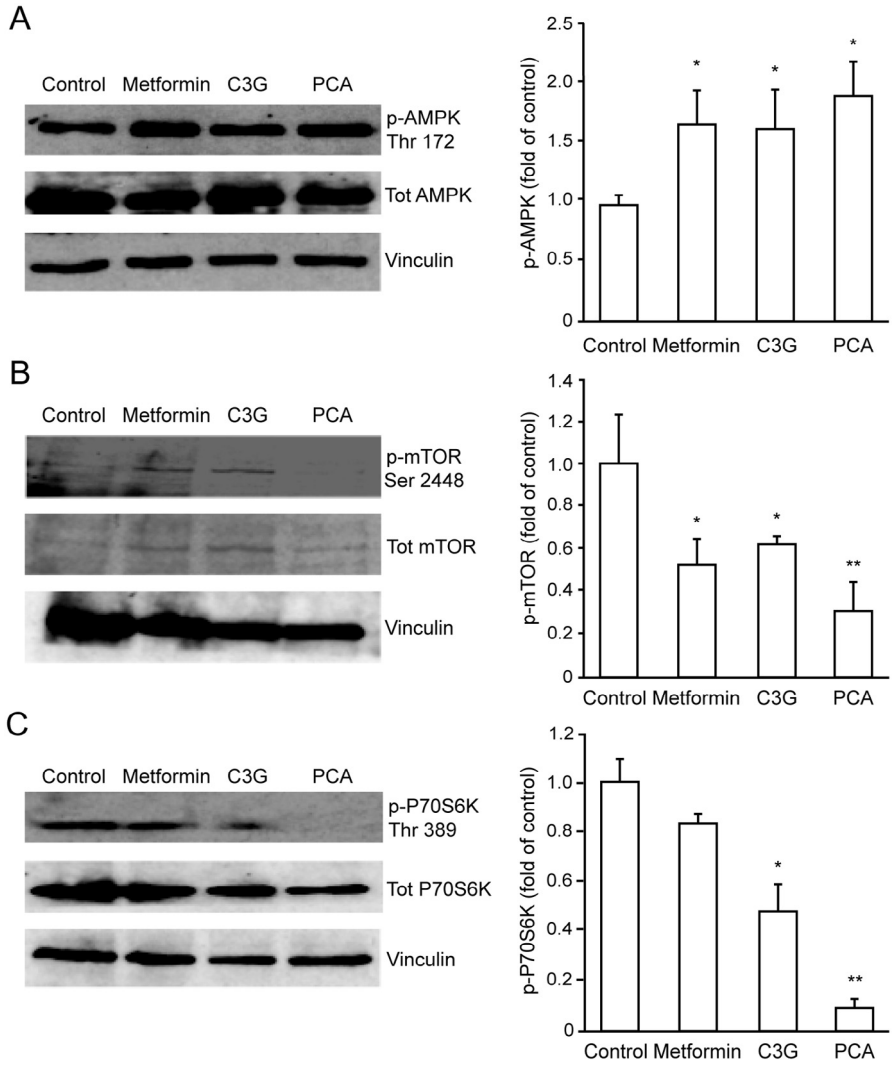

Fig. 1 – C3G and PCA activate AMPK and inhibit mTOR and S6K in BNL cells.
Representative images (left panels) and densitometric results (right panels) of WB analysis for AMPK phosphorylation at Thr 172 (A), mTOR phosphorylation at Ser 2448 (B) and P70S6K phosphorylation at Thr 389 (C) on protein extract from BNL cells. Results of the graphs are the ratio of averages with Control from three independent experiments with ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$ *vs.* Control.

suggesting that these compounds activated AMPK primarily by promoting its phosphorylation by LKB1.

LKB1 activation of AMPK maintains energy homeostasis ultimately increasing ATP levels. Consistently, the treatment of BNL for 6 hours with 50 μM C3G or PCA increased ATP levels (Fig. 3D) without affecting mitochondrial respiration rate (Fig. 3E).

These findings indicate that C3G and PCA are indirect activators of AMPK in BNL cells.

3.4. *C3G and PCA induce glucose tolerance in mice and the expression of glucose transporters in the liver*

A large body of literature has shown that AMPK is central in the control of glucose metabolism (Viollet et al., 2009; Zhang, Zhou, & Li, 2009) and its activation seems required for the antidiabetic effect of the widely prescribed drug metformin (Shaw et al., 2005; Zhou et al., 2001). To analyse the ultimate effect of C3G and PCA on glucose homeostasis we performed GTT in mice after acute administration of C3G and PCA. As reported in Fig. 4A the administration of both C3G and PCA at a concentration of 1 g/kg induced a slight drop (not statistically significant) in basal glucose level (time 0) as equivalent to metformin. At 20, 40 and 60 min upon intraperitoneal administration of glucose (2.0 g/kg), there was significant ($p < 0.01$ at 20 min, $p < 0.05$ at 40 and 60 min) decrease in glucose level in C3G and PCA treated mice when compared to control mice (treated by water only). The similar results were obtained for the positive control metformin. Notably, C3G, PCA and metformin had comparable effects on the rise of insulin levels measured by ELISA in blood samples during the GTT (Fig. 4B).

AMPK activation stimulates expression and translocation to the plasma membrane of the glucose transporters Glut 1 and Glut 4 to enhance glucose entry into the cell (Hayashi et al.,

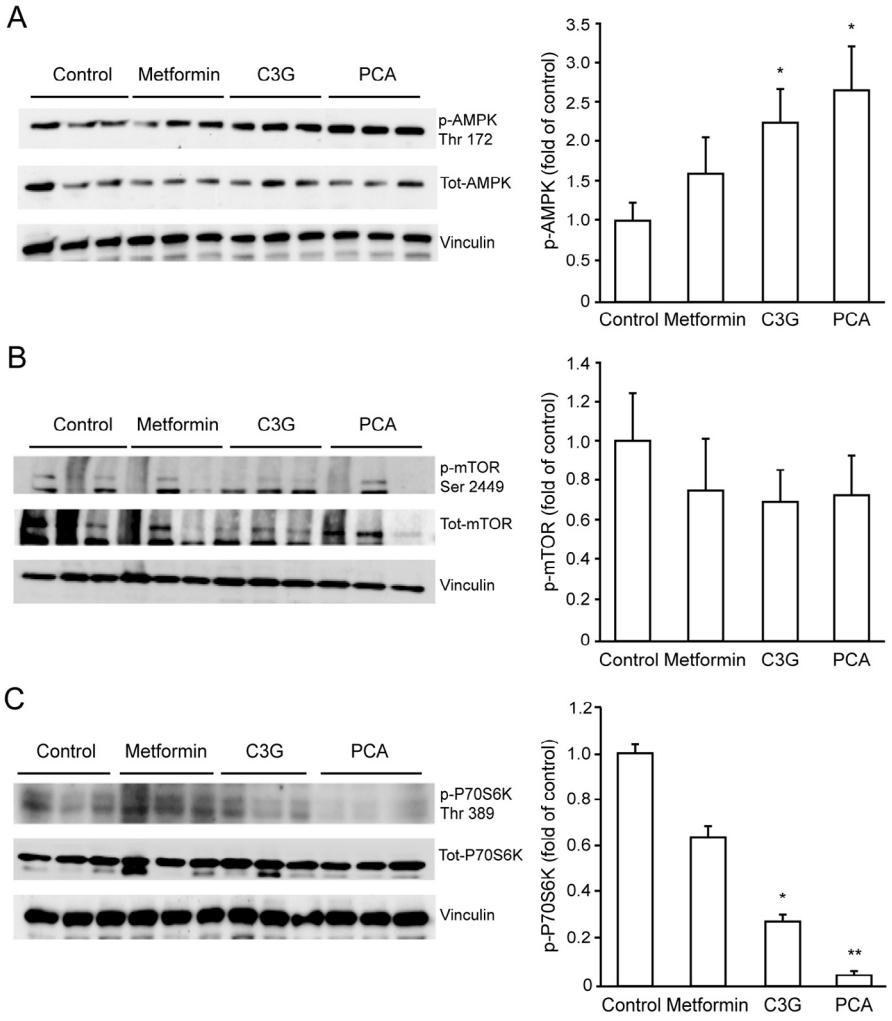

Fig. 2 – C3G and PCA activate AMPK and inhibit mTOR and S6K in liver tissue.
Representative images (left panels) and densitometric results (right panels) of WB analysis for AMPK phosphorylation at Thr 172 (A), mTOR phosphorylation at Ser 2448 (B) and P70S6K phosphorylation at Thr 389 (C) on protein extract from mouse livers. Results of the graph are the ratio of averages with Control from two independent experiments with ± SEM. *$p < 0.05$, **$p < 0.01$ *vs.* Control.

2000). We measured Glut 1 and 4 expressions in liver extracts from mice treated with C3G and PCA by WB. The immunoblots results indicated that C3G and PCA treatments were associated with significant enhancement of Glut 1, 4.8 ± 0.2 ($p < 0.01$) fold in C3G treated and 6.0 ± 0.2 ($p = 0.002$) fold in PCA treated samples compared to control (Fig. 4C), but not as such of Glut 4 protein levels, significant only for PCA treatment (3.7 ± 0.8 fold in compared to control $p < 0.05$) (Fig. 4D) suggesting that increasing glucose transporters expression play a role in improved glucose tolerance by C3G and PCA.

3.5. *PCA improves glucose tolerance and insulin sensitivity in obese mice*

To validate the improving effect of C3G and PCA on glucose tolerance in pre-diabetic condition we performed GTT and ITT in mice that were fed 6 months HFD. As expected, the weight gained by these mice almost doubled the weight of aged matched mice SD fed mice (42.1 ± 1.4 g in SD fed group *vs.* 27.8 ± 0.5 g in HFD fed group; $p < 0.01$). Circulating free fatty acids, triglycerides and cholesterol resulted also significantly increased upon HFD (respectively 0.52 ± 0.16 meq/dl, 56 ± 12 mg/dl, 49 ± 8 mg/dl in SD fed group and 1.36 ± 0.28 meq/dl, 162 ± 35 mg/dl, 131 ± 24 mg/dl in HFD fed group; $p < 0.01$ for all three parameters) as well as insulin levels (1.35 ± 0.3 ng/dl in standard diet fed group and 1.45 ± 0.3 ng/dl in HFD fed group; $p < 0.01$) whereas basal glycaemia was only slightly, though not significantly, increased in HFD fed mice. In these obese mice, the pre-treatment with metformin in or C3G (1 g/kg) 1 hour before GTT did not affect the curve of glucose levels (Fig. 5A) however 1 g/kg PCA pre-treatment significantly reduced glycaemia at 40 and 60 min after glucose injection and the overall area under the GTT glycaemic curve (Fig. 5A). Furthermore, ITT on mice fed and treated with metformin, C3G or PCA revealed that the administration of PCA amplified glucose drop (Fig. 5B) indicating that PCA particularly improved insulin

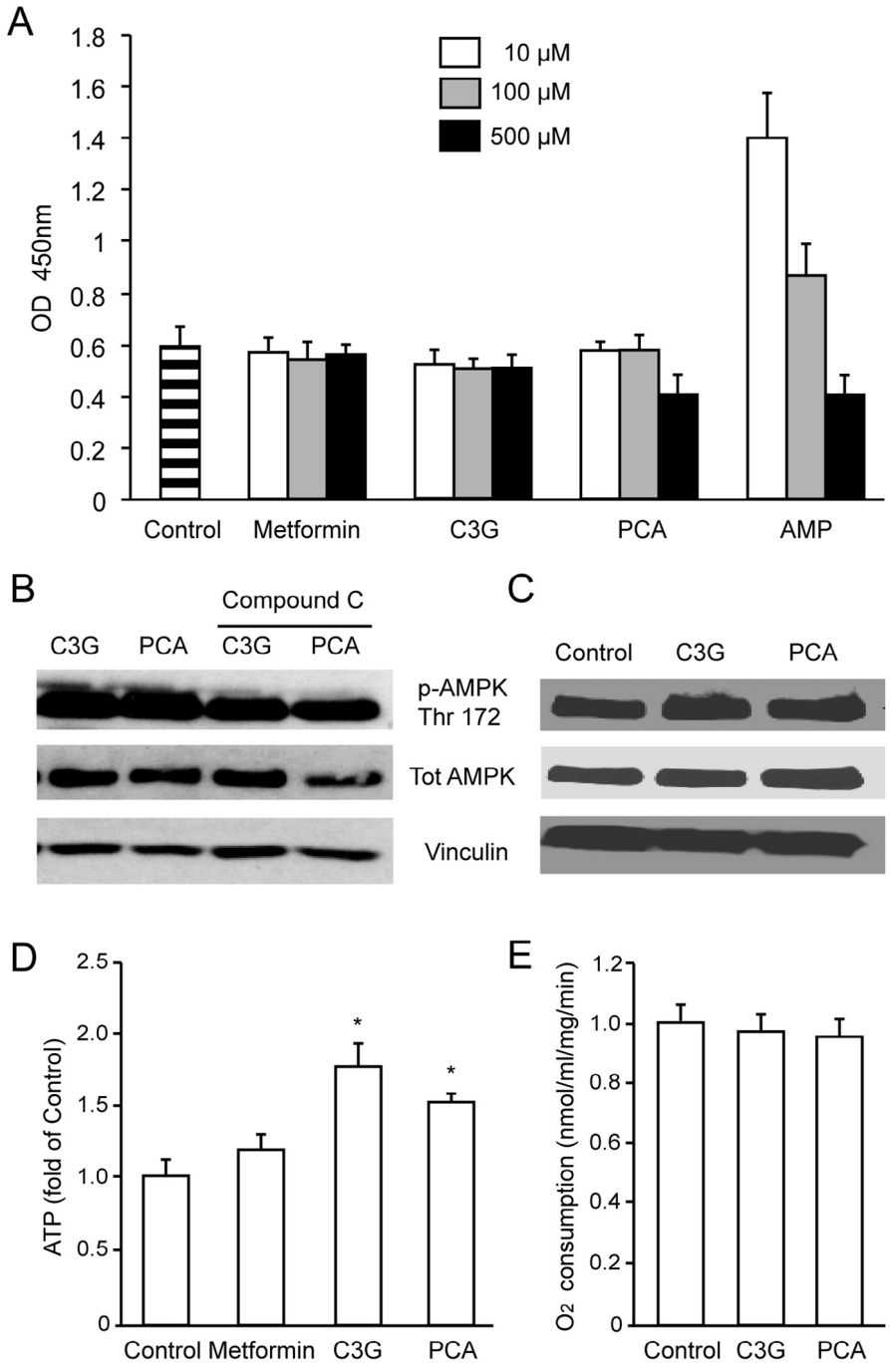

Fig. 3 – C3G and PCA are indirect activators of AMPK. (A) Spectrophotometric quantification of AMPK kinase activity as determined by an Elisa method. AMP was used as a positive control for AMPK activation. Data are means ± SEM from 3 independent experiments. (B) WB analysis of phosphorylation of Thr 172AMPK in BNL cells treated with 0.1 mM C3G, PCA and 20 µM Compound C for 6 hours. (C) WB analysis for AMPK phosphorylation in Hela cells were treated with 0.5 mM C3G or PCA for 6 hours. (D) Intracellular ATP levels in BNL cells treated for 6 hours with the 0.5 mM concentrations of C3G and PCA. (E) Respiration of isolated mitochondria from mouse liver in the presence of 0.1 mM PCA and C3G. Mean ± SEM values of 3 independent measurements are shown. *$p < 0.05$ *vs.* Control.

sensitivity in obese mice. Finally, the treatment for 2 months of mice fed HFD with blood orange juice, enriched in C3G, but not with blond orange juice, without anthocyanins, as substitute of drinking water improved significantly insulin sensitivity as revealed by the ITT reported in Fig. 5C.

4. Discussion

The potential of polyphenols in controlling glycaemia is currently under intensive study. However, the biological properties

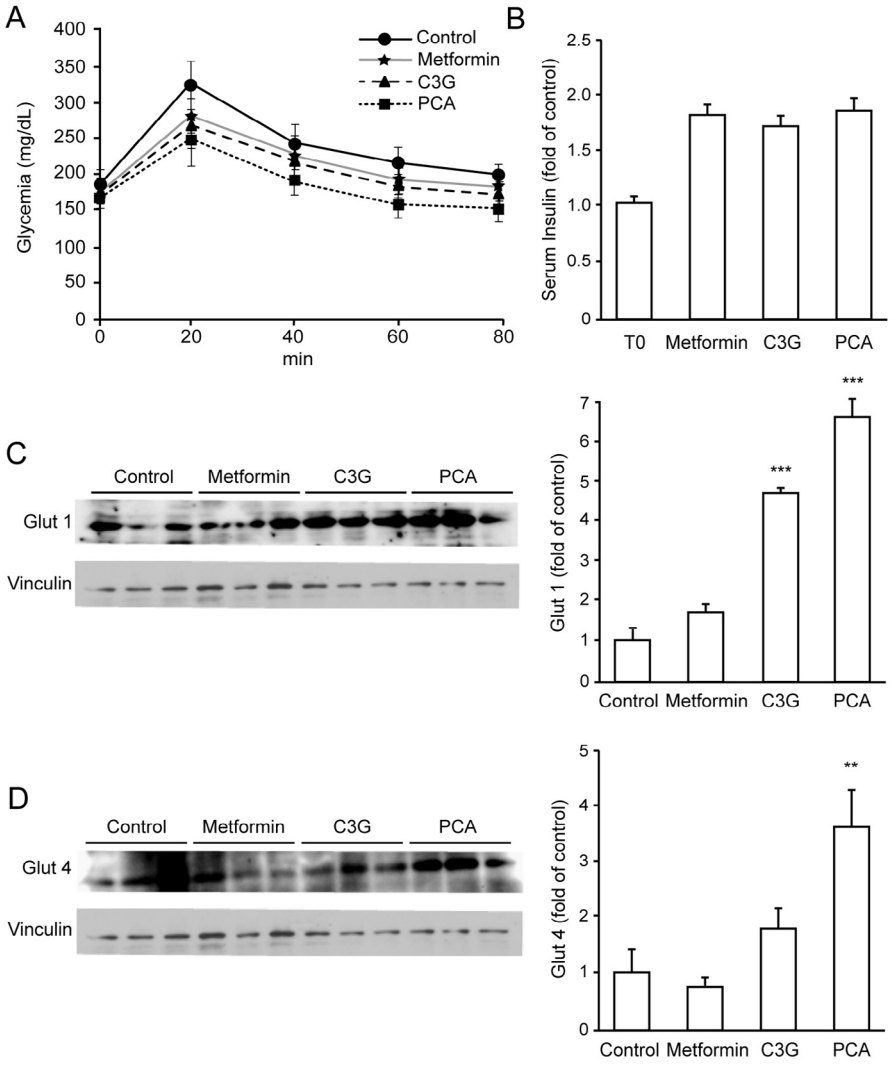

Fig. 4 – C3G and PCA improve glucose homeostasis in mice. (A) Blood glucose levels measured during the GTT of mice treated with vehicle (Control), metformin, C3G or PCA as described in methods and insulin levels (B) at 20 min of the GTT. Values are average ± SEM (*n* = 6 mice/group). Representative images (left panels) and densitometric results (right panels) of WB analysis for Glut 1 (C) and Glut 4 (D) expression in liver protein extracts from mice treated or not with metformin, C3G or PCA. Values are means ± SEM (n = 3). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ *vs.* Control.

of anthocyanins have nearly always been studied *in vitro* by using their native form, which appears quite inappropriate because of their in vivo extensive and rapid biotransformation after ingestion (Kay et al., 2009; Vitaglione et al., 2007). Indeed, the native forms of anthocyanins are slightly present in the bloodstream (Vitaglione et al., 2007), and they might be metabolites such as PCA, which likely reach tissues and may exert biological effects. So far, PCA has been poorly investigated because of its low concentration in foods.

Here, we have shown that purified C3G from blood orange and PCA activate AMPK and down regulate mTOR/S6K pathway in hepatic cell culture at millimolar concentration range, though higher with respect to that reached *in vivo* (Giordano et al., 2012). Indeed, the effects of C3G and PCA in BNL cells, below 0.1 mM, on AMPK/TOR/S6K pathway were not consistent. Activation of AMPK and S6K were confirmed in the liver upon one hour of oral administration of C3G and PCA to mice. At that time point the concentration of anthocyanin in the mouse plasma peaked around 0.1 μM as evaluated by HPLC analysis of upon solid phase extraction with HLB SPE columns (Titta et al., 2010). Why the effective dosage is different *in vitro* and *in vivo* models is unclear. Cell culturing could alter the network of kinases leading to a reduced sensitivity of AMPK pathway to anthocyanins, culture medium may affect degradation of anthocyanins and/or prevent the formation of bioactive derivatives whereas *in vivo* polyphenols might concentrate at cellular level in the tissue microenvironment and/or a specific hyperactive metabolite or a synergising mixture of them may be generated.

PCA is an important derivative of cyanidin, showing anti-inflammatory, anti-hyperglycaemic and neuroprotective activities (Masella et al., 2012), that in our setting reproduces the effect of C3G on AMPK, TOR and S6K yet at millimolar range.

Regardless, administration *in vivo* of C3G and PCA induced a significant increase in AMPK phosphorylation in the liver, but not in WAT and muscle tissue, as observed with a pharmacological dose of Metformin, the most common AMPK-activator

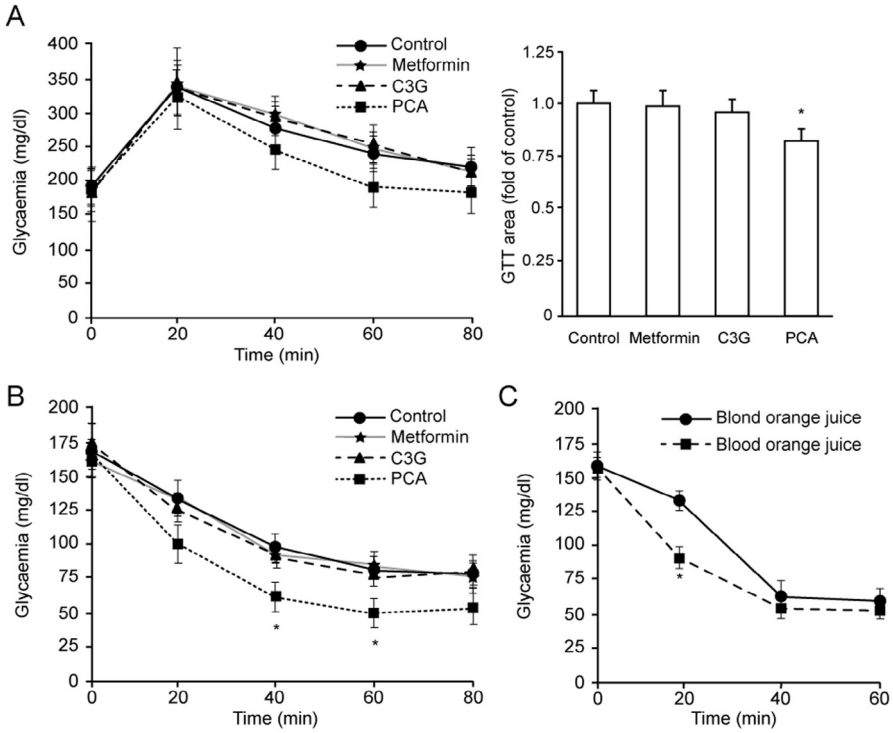

Fig. 5 – PCA improves glucose tolerance and insulin sensitivity in obese mice. (A) GTT of obese mice treated with vehicle (Control), metformin, C3G or PCA and the corresponding values of the areas under the glycaemic curves (left graph). (B) ITT of obese mice treated with vehicle (Control), metformin, C3G or PCA. (C) ITT of mice fed HFD *and drinking blood or blond orange* juices. Values are average ± SEM (*n* = 6 mice/group). *p < 0.05. Control.

anti-diabetic drug. From this point of view, the polyphenol concentrations tested in our experiments, although higher than that reported after ingestion of food rich in anthocyanin, can provide significant information.

Furthermore, PCA administration was more effective on glucose tolerance in mice fed standard diet and substantially it showed a unique improvement of glucose tolerance and insulin sensitivity in obese mice. Whether this peculiar efficacy of PCA with respect to C3G is the consequences of lower absorption and availability or of gut metabolism of C3G in the mice fed high fat diet is unclear.

AMPK is activated by a wide variety of xenobiotics, many being plant products reputed to have health benefits that are either 'nutraceuticals' or have been used in traditional medicines as anti-diabetic. Metformin and the other anti-diabetic drugs thiazolidinediones are believed to act, at least in part, by indirectly activating AMPK (Hawley et al., 2010). Resveratrol also indirectly activates AMPK by increasing cytosolic calcium levels or by facilitating AMP-dependent activation of AMPK (Vingtdeux et al., 2010). Several small molecules that bind and directly activate AMPK, such as AICAR, A-769662, or PT1, were identified (Zhang et al., 2009) These molecules bind either to the regulatory subunit or to the catalytic subunit of AMPK to directly activate the kinase (Zorn & Wells, 2010) C3G and PCA did not activate recombinant AMPK *in vitro* (Fig. 3A), suggesting that these compounds did not bind nor directly activated AMPK. Treatment with compound C, which is an inhibitor of AMPK, has no effect on AMPK phosphorylation induced by C3G and PCA suggesting that these polyphenols act upstream from AMPK. Then, as there is no effect of C3G and PCA in Hela cells lacking the AMPK upstream activator LKB1 (Fig. 3C), these polyphenols act through LKB1.

Since mild perturbation of mitochondrial function has been shown to be associated with activation of AMPK (Hayashi et al., 2000), the effect of C3G and PCA on mitochondrial functions were examined. Treating intact cells or isolated liver mitochondria with C3G and PCA has no effect on mitochondrial trans-membrane potential (data not shown) or respiration (Fig. 3E). Consistently, cellular levels of ATP levels increased after treating with C3G and PCA. Further investigation will be needed to identify the precise mechanism of AMPK activation by C3G and PCA.

Recently, PCA was reported to induce AMPK in vascular smooth muscle cells (Lin, Ou, Chang, Chan, & Wang, 2015) whereas polyphenol extract of *Hibiscus sabdariffa* containing PCA was known to lower glycaemia and improve insulin sensitivity in a rat model of T2D diabetes (Peng et al., 2011). Indeed, many food sources of C3G and/or PCA other than blood oranges have been associated with anti-diabetic activity including green tea (Wang et al., 2014), pigmented rice (Deng et al., 2013), pomegranate juice (Medjakovic & Jungbauer, 2013) and berries (Wedick et al., 2012) thus supporting the functional value of food sources of C3G and PCA to improve the dietary treatment of T2D.

5. Conclusions

Blood orange juice is a dietary source of C3G and PCA. Here, we have shown that C3G and PCA activate the AMPK/mTOR/S6K

pathway in cultured cell and *in vivo* models. The oral administration of C3G and PCA in mice had significant beneficial effects on glucose tolerance accompanied by high expression of glucose transporters glut 1 and glut 4 in the liver. In particular, PCA improved glucose tolerance and insulin sensitivity in obese mice that model early stage of T2D. Consistently, the consumption of blood orange juice improved insulin sensitivity in mice fed HFD.

Therefore, PCA deserves great nutritional interest as the main human metabolite of C3G, which in turn the most representative dietary anthocyanins. Finally, our study offers a new clue to the possible use of anthocyanins dietary sources against metabolic syndrome.

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