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The effect of cyanidin-3-O-β-glucoside and peptides extracted from yoghurt on glucose uptake and gene expression in human primary skeletal muscle myotubes from obese and obese diabetic participants

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ABSTRACT

Incidence of type II diabetes mellitus (T2DM) is rapidly increasing worldwide. This study aimed to investigate whether cyanidin-3-O-β-glucoside (C3G), or peptides with angiotensin converting enzyme (ACE) inhibitory activity, alone or in combination, alter glucose regulation in human primary myotubes derived from obese and obese T2DM participants. In the obese group, both low and high concentration of peptides and the combination of these peptides with high C3G concentration significantly enhanced glucose uptake in the presence or absence of insulin, and high concentration of peptide alone and its combination with low C3G down-regulated the mRNA expression of angiotensin II receptor, type 1 (AGTR-1), and up-regulated the mRNA expression of insulin receptor substrate 1 (IRS-1), and glucose transporter 4 (GLUT4). However, only high peptide concentration increased glucose uptake in the absence of insulin in the obese T2DM group, and the expression of AGTR-1 was decreased with high peptide and its combinations of C3G.

1. Introduction

Skeletal muscle is a key site for insulin mediated glucose disposal in mammals and is therefore an important regulator of whole body glucose homeostasis. In this tissue, insulin binds to the insulin receptor (IR) and activates IR substrates (IRS-1 and IRS-2) (Krishnapuram et al., 2013). Activated IR substrates trigger downstream activation of the lipid kinase phosphatidylinositol-3 kinase (PI3K), and finally stimulate the translocation of glucose transporter 4 (GLUT4) to facilitate the transport of glucose into cells (Guo et al., 2008; Johnston, Pirola and Van Obberghen, 2003). Additionally GLUT4 translocation can also be activated through the cellular energy sensor 5′adenosine monophosphate-activated protein kinase (AMPK) which regulates cell metabolism and integrates nutritional and hormonal signals (Alkhateeb and Qnais, 2017; Naimi, Vlavcheski, Murphy, Hudlicky and Tsiani, 2017). Activation of AMPK has been viewed as a targeted approach to increase glucose uptake by the skeletal muscle and therefore improve blood glucose homeostasis. Impaired glucose uptake into skeletal muscle is attributable to insulin resistance, which is associated with several defects within the insulin signalling cascade, namely reduced IR substrates and PI3K response to insulin stimulation (Dos Santos, Moreli, Tewari and Benite-Ribeiro, 2015). Insulin resistance can be associated with obesity and plays a key role in the development of type 2 diabetes mellitus (T2DM) (Barazzoni, Gortan Cappellari, Ragni and Nisoli, 2018; Bouzakri, Koistinen and Zierath, 2005; Deshmukh, 2016).

Cyanidin-3-O-β-glucoside (C3G) is a predominant bioactive anthocyanin compound found in many edible plants and has been reported to be protective against T2DM by attenuating multiple disorders *in vivo* and *in vitro* (Guo, Guo, Jiang, Li and Ling, 2012; Guo et al., 2012; Scazzocchio, et al.. , 2011; Wang, Zhao, Wang, Huo and Ji, 2016). It has been found that C3G increased glucose uptake in human skeletal muscle cells due to its strong antioxidant activity (Ho, Kase, Wangensteen and Barsett, 2017). C3G derived from black soybeans ameliorated T2DM in db/db mice through the induction of differentiation of 3T3-L1 preadipocytes into smaller and insulin-sensitive adipocytes, which subsequently resulted in activation of insulin signalling and increased

Abbreviations: ACE, angiotensin converting enzyme; AGTR-1, angiotensin II receptor, type 1; AMPK, 5′adenosine monophosphate-activated protein kinase; Ang II, angiotensin-II; BCA, bicinchoninic acid; C3G, cyanidin-3-O-β-glucoside; DEPC, diethylpyrocarbonate; DOG, deoxyglucose; FOXO1, forkhead box protein O1; GAPDH, glyceraldehydes-3-phosphate dehydrogenase; GLUT1, glucose transporter 1; GLUT4, glucose transporter 4; IR, insulin receptor; IRS-1, insulin receptor substrate-1; IRS-2, insulin receptor substrate-2; PI3K, phosphatidylinositol-3 kinase; PPAR-α, peroxisome proliferator–activated receptor-alpha

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glucose uptake (Matsukawa, Inaguma, Han, Villareal and Isoda, 2015). Another *in vitro* study using L6 myotubes showed that C3G significantly induced AMPK activation and enhanced glucose uptake into the cells (Kurimoto et al., 2013). However, no information is available on the effect of C3G on glucose metabolism in human skeletal muscle cells, and the molecular mechanism/s underlying this action also remains unclear.

Angiotensin-converting enzyme (ACE) is a primary enzyme in the regulation of blood pressure through converting angiotensin-I into a potent vasoconstrictor, angiotensin-II (Ang II). Subsequently, angiotensin-II induces the release of aldosterone and therefore increases the sodium retention and blood pressure (Muro Urista, Alvarez Fernandez, Riera Rodriguez, Arana Cuenca and Tellez Jurado, 2011). The blood pressure regulator ACE has also been shown to influence glucose uptake. Angiotensin- II (Ang II) has been shown to antagonize insulin-independent contraction-induced glucose uptake in skeletal muscle tissue (Rao, 1994) via the Ang II type 1-receptor (AGTR-1) (Jones and Woods, 2003). The chronic effects of Ang II on myocytes are associated with a reduced protein expression of GLUT4, suggesting limited availability of the glucose transporter to be partially responsible for the restricted glucose uptake (Shiuchi et al., 2004). In addition, Ang II inhibits insulin-mediated actions at multiple levels of the insulin signalling pathway including IR, IR substrates and PI3K (Igarashi, Hirata, Nozaki, Kadomoto-Antsuki and Tominaga, 2007; Shiuchi et al., 2004). Thus ACE inhibitors or AGTR-1 blockers could enhance whole-body and skeletal muscle insulin sensitivity, which is associated with increased expression of GLUT4 in skeletal muscle (Henriksen, Jacob, Kinnick, Teachey and Krekler, 2001). It has previously been determined that the ACE inhibitor temocapril improves insulin resistance and glucose intolerance by increasing glucose uptake in skeletal muscle at least in part through GLUT4 translocation (Shiuchi et al., 2002). AGTR-1 blockade also increases insulin sensitivity and glucose uptake in skeletal muscle of KK-Ay mice, via stimulation of the insulin signalling cascade and increasing GLUT4 translocation to the plasma membrane (Shiuchi et al., 2004). However, no studies have elucidated the underlying mechanism of yoghurt peptides with high inhibition of ACE activity extracted from yoghurt in improving glucose uptake in human skeletal muscle cells.

We therefore aimed to investigate whether, in isolation and/ or in combination, C3G and peptides with ACE inhibitory activity enhanced glucose uptake, and to understand the related molecular mechanisms of the action. We hypothesized that the combinations of C3G and peptides with ACE inhibitory activity would improve insulin sensitivity by regulating glucose disposal in human primary skeletal muscle myotubes derived from obese or obese T2DM individuals.

2. Material and methods

2.1. Material and regents

HPLC grade C3G was provided by Polyphenols AS (Sandnes, Norway). Pierce™ bicinchoninic acid (BCA) Protein Assay Kit, Diethylpyrocarbonate (DEPC) water, α-MEM, amphotericin B, horse serum, fetal bovine serum (FBS), and trypsin/EDTA were obtained from Life Technologies Australia Pty Ltd (Mulgrave, VIC, Australia). iQ™ SYBR Green and iScript™ cDNA Synthesis Kit were purchased from Bio-Rad Laboratories Pty Ltd (Gladesville, NSW, Australia). Liquid scintillation cocktail and deoxy-D-glucose, 2-[1,2-3H (N)]- were purchased from Perkin-Elmer, Inc. (Glen Waverley, VIC, Australia). All other chemicals, unless otherwise specified, were obtained from Sigma-Aldrich Pty. Ltd. (Sunshine, VIC, Australia).

Peptides were extracted from yoghurt fermented by *Lactobacillus helveticus* 881315 in the presence of Flavourzyme for 12 h. ACE inhibitory activity of peptides was determined using a reversed-phase-HPLC system (RP-HPLC, from Varian Analytical Instruments, Santa Clara, CA, USA) and the IC_{50} value of peptides was 1.47 ± 0.04 mg/mL. The details on the process of fermentation, peptide extraction and determination of ACE inhibitory activity have previously been reported (Min, Fatah, Michael, Andrew and Xiao, 2017). Subsequently, peptides derived from the same batch (with an IC_{50} value of 1.47 ± 0.04 mg/mL) as we have previously reported were used in the present cell culture study (Min, et al., 2017).

Table 1
Participant characteristics.

	Participants	
	Obese	Obese Type 2 Diabetes Mellitus
n (F/M)	8/2	8/2
Age (years)	49 ± 4	53 ± 2
BMI (kg/m^2)	40.42 ± 1.64	42.80 ± 2.38
Fasting plasma glucose (mmol/L)	5.02 ± 0.13	8.61 ± 0.76*
HbA1c (%)	5.59 ± 0.09	7.82 ± 0.47*
Plasma insulin (μU/L)	10.17 ± 1.64	21.62 ± 2.53*
Cholesterol (mmol/L)	5.74 ± 0.52	5.01 ± 0.37
Fasting Triglyceride (mmol/L)	1.75 ± 0.31	2.36 ± 0.46

Values are expressed as means ± SEM.
* Indicated a significant difference between obese and obese T2DM groups ($p < 0.05$).

2.2. Participants

10 obese (eight female and two male), and 10 obese T2DM (eight female and two male) participants undergoing abdominal surgery were recruited for additional tissue collection (Table 1). After fasting for 12–18 h, general anesthesia was induced with a short-acting propofol and maintained by a fentanyl and rocuronium volatile anesthetic mixture, and a biopsy of rectus abdominus muscle was removed as previously described (Chen et al., 2005). This research was approved by the Human Ethics Research committee of Victoria University (HRE08-158), and all participants gave written informed consent.

2.3. Human primary skeletal muscle myotubes

Human primary skeletal muscle cell culture was established as previously described (Blau and Webster, 1981; Gaster, Beck-Nielsen and Schroder, 2001). Cells were thawed at passage four and grown to 70% confluence in growth media [α-MEM supplemented with 10% FBS (vol/vol), 0.5% penicillin (vol/vol) and 0.5% amphotericin B (vol/vol)]. Cells were then detached and resuspended in the growth medium and seeded on six-well and twelve-well plates (Greiner, Longwood, FL) coated with essential coating matrix (ECM) gel. Once at 80% confluence, the growth medium was replaced by the differentiation media [α-MEM, 2% horse serum, 0.5% penicillin (vol/vol) and 0.5% amphotericin B (vol/vol)] for 6 days for subsequent experimentation.

2.4. Glucose uptake bioassay

Glucose uptake assay in human primary skeletal muscle myotubes was established according to previously described methods (Boue, Daigle, Chen, Cao and Heiman, 2016) with minor modification. Following differentiation cells were treated with C3G (10 and 100 μM) and peptides with ACE inhibitory activity (150 μg/mL and 1500 μg/mL) in isolation and combination in the presence of serum-free medium for 24 h. At the end of the treatment period the cells were treated in the presence or absence of insulin 100 nM at 37 °C for 30 min and supplemented with 2-deoxyglucose (DOG)/2-[3H]DOG (10 μM final concentration, 1 μCi/well) for 15 min at 37 °C. The reactions were stopped by washing cells with cold PBS. Cells were then solubilized by the addition of 0.5 mL of 0.3 M NaOH. An aliquot of 400 μL was utilized for the determination of glucose transport activity by scintillation counter

(Tri-Carb 3180TR/SL; Perkin-Elmer, Inc.) and expressed as picomoles of DOG taken up per minute per milligram of total protein. The remaining solution was utilized for determination of protein concentration via BCA assay using a Pierce™ BCA Protein Assay Kit based on the previous method (Bainor, Chang, Mcquade, Webb and Gestwicki, 2011). Absorbance was read at 562 nm with a Varioskan™ LUX Multimode Microplate Reader (Thermo Fisher Scientific Laboratory Equipment, Hudson, United States). A standard curve was prepared and protein content for each sample was calculated.

2.5. RNA extraction

Following treatments, human primary myotubes were lysed using TRIzol and then stored at −80 °C prior to total cellular RNA extraction according to previously established methods (Mcainch et al., 2006). Total RNA was quantified using NanoDrop spectrometry (NanoDrop 2000, Thermo Fisher Scientific, Wilmington, DE) at 260 nm. 0.5 µg of total RNA was reverse transcribed into cDNA using iScript™ cDNA Synthesis Kit according to the manufactures instructions using a MyCycler™ Thermo Cycler (Bio-rad Laboratories, Hurcules, CA).

2.6. Real-time polymerase chain reaction (PCR) analysis

'Real-time' PCR was conducted using MyiQ™ multiplex 'real-time' PCR detection system (Bio-Rad Laboratories, Hercules, CA) with iQ™ SYBR Green Supermix. Forward and reverse oligonucleotide primers (Table 2) for the gene of interest were designed using OligoPerfect™ Suite (Invitrogen, Carlsbad, CA). Selective gene homology for genes of interest was confirmed with BLAST (Basic Local Alignment Search Tool, National Centre for Biotechnology Information, Bethesda, MD). To compensate for variations in RNA input amounts and reverse transcriptase efficiency, mRNA abundance of the housekeeping genes, cyclophilin and glyceraldehydes-3-phosphate dehydrogenase (GAPDH) were also measured. The 'real-time' PCR cycling parameters were as follows: initial denaturation and enzyme activation at 95 °C for 3 min, followed by 40 cycles of denaturation at 95 °C for 15 s, annealing and extension at 60 °C for 45 s. Relative changes in mRNA abundance was normalised to the average of two housekeeping genes (cyclophilin and GAPDH) and then quantified using the $2^{-\Delta\Delta CT}$ method. Treatment groups were expressed relative to control treatment and all experiments were repeated at least three times.

2.7. Statistical analysis

All results were expressed as mean ± standard error of the mean (SEM) for each measurement (n = 8–10). Two-way ANOVA was performed to determine the differences of glucose uptake rate between the presence and absence of insulin in all treatments using GraphPad Prism 7 (GraphPad Software, Inc, La Jolla, CA, USA). Student *t*-test was used to analyse the significant difference in characteristics of participants between obese and obese T2DM groups. One-way ANOVA was performed to analyse the significant differences in the mRNA expression of genes between control and treatments. Fisher's (least significant difference; LSD) test was used to differentiate significant differences amongst the treatment groups. $P < 0.05$ was considered significant.

3. Results

3.1. Effect of C3G and peptides with ACE inhibitory activity on glucose uptake in human primary skeletal muscle myotubes of obese and obese T2DM participants

As shown in Fig. 1A, both low and high C3G concentrations (10 and 100 µM), and the combinations of low C3G with peptides (both low and high concentrations) did not alter glucose uptake in the absence of insulin in human skeletal muscle myotubes from obese participants. However, glucose uptake in human myotubes from obese participants treated with high C3G (100 µM) with insulin stimulation was significantly increased 1.41 fold ($P = 0.044$), compared to cells treated with insulin alone. Furthermore, high C3G concentration (100 µM) combined with peptides at the concentrations of 150 µg/mL and 1500 µg/mL in the presence or absence of insulin significantly enhanced glucose uptake ($P < 0.001$), compared with their respective controls. Furthermore, compared with high C3G concentration alone, the combinations of C3G and peptides significantly increased glucose uptake ($P < 0.001$, Fig. 1A) in the absence of insulin. The high peptide concentration (1500 µg/mL) alone resulted in the highest glucose uptake with 1.87 ± 0.59 fold being recorded in the absence of insulin, compared with the control (Fig. 1A). Similarly, neither low C3G concentration alone nor its combination with low peptide altered glucose uptake in the presence of insulin in the obese group, compared with the control (Fig. 1A). In contrast, glucose uptake was improved when treated with low C3G concentration combined with high peptide concentration ($P = 0.011$, Fig. 1A) in the presence of insulin, compared to control. In addition, the other treatments including high C3G concentration, low and high peptide concentrations alone and their combinations resulted in a significant increase in glucose uptake in the presence of insulin, compared with the control in the obese group.

Fig. 1B illustrates that, only high peptide (1500 µg/mL) significantly enhanced glucose uptake (by 1.39 ± 0.23 fold) in the absence of insulin in the obese T2DM group, compared with the control group ($P = 0.002$). Glucose uptake was not altered by any of the treatments in the presence of insulin in the obese T2DM group.

Table 2
Human primer sequences used for 'Real-time' PCR analysis.

Gene	Forward Primer (5′ – 3′)	Reverse Primer (5′ – 3′)
AGTR-1	CTGATGCCATCCCAGAAAGT	CTTCCAGCTTTGGGACAATC
AMPK-α	AACTGCAGAGAGCCATTCACTTT	GGTGAAACTGAAGACAATGTGCTT
Cyclophilin	CATCTGCACTGCCAAGACTGA	TTCATGCCTTCTTTCACTTTGC
FOXO1	TCATGGATGGAGATACATTGGATT	TCCTGCTGTCAGACAATCTGAAG
GAPDH	CAA CGA CCA CTT TGT CAA GC	TTA CTC CTT GGA GGC CAT GT
GLUT1	GGGCCAAGAGTGTGCTAAAG	TGCCGACTCTCTTCCTTCAT
GLUT4	CACCCTCACCACCCTCTG	CTTTTCCCAAGCCACTG
IRS-1	GTTTCCAGAAGCAGCCAGAG	TGAAATGGATGCATCGTACC
IRS-2	ACGCCAGCATTGACTTCTTGT	TGACATGTGACATCCTGGTGATAA
PI3K	GGAAGCAGCAACCGAAACAA	TTCGCCGTCCACCACTACA
PPAR-α	GAAGCTGTCCTGGCTCAGAT	GGGGACCACAGGATAAGTCA

AGTR-1, angiotensin II receptor type 1; AMPK-α, 5′ AMP-activated protein kinase alpha; FOXO1, Forkhead box protein O1; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; GLUT1, Glucose transporter 1; GLUT4, Glucose transporter 4; IRS-1, Insulin receptor substrate 1; IRS-2, Insulin receptor substrate 2; PI3K, Phosphoinositide 3-kinase; PPAR-α, Peroxisome proliferator-activated receptor alpha.

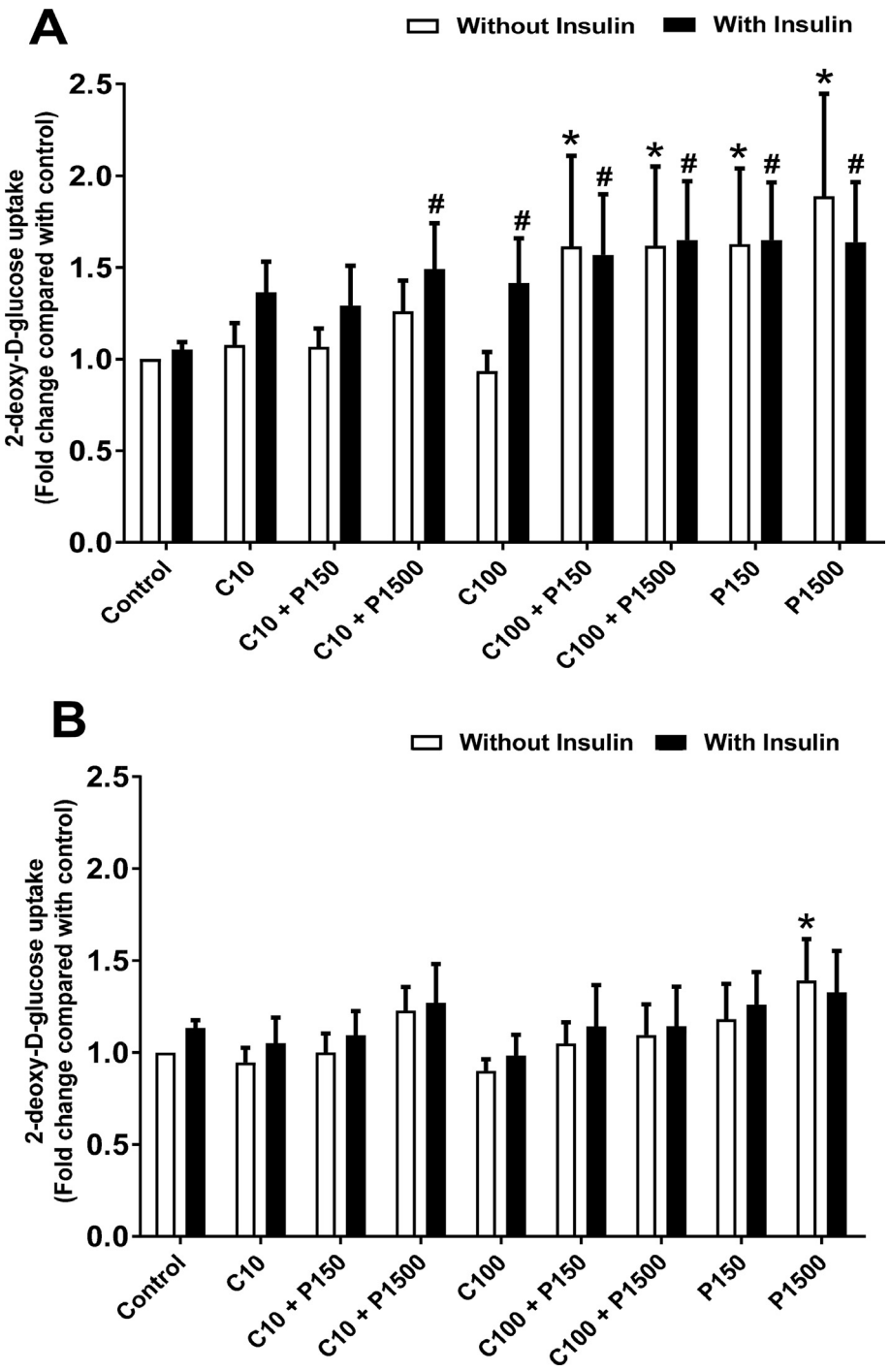

Fig. 1. The effect of C3G and peptides on glucose uptake in human primary skeletal muscle myotubes derived from obese participants (A) and obese T2DM participants (B). Cells were treated with BSA (as a vehicle control), 10 μM or 100 μM of C3G, 150 μg/mL or 1500 μg/mL of peptides and combinations of C3G/peptides in the presence or absence of insulin for 24 h. Data was normalised to control with or without insulin, respectively. Data was expressed as mean ± SEM (n = 10). *$P < 0.05$ compared with control without insulin stimulation; #$P < 0.05$ compared control with insulin stimulation.

3.2. Expression of AGTR-1, GLUT4, IRS-1 and forkhead box protein O1 (FOXO1) in primary human skeletal muscle myotubes of obese and obese T2DM participants

In this study, we investigated the effects of C3G and peptides in isolation and in combination on the expression of multiple genes associated with obesity and T2DM including AGTR-1, GLUT4, IRS-1 and FOXO1 by 'Real-Time' PCR analysis. As shown in Fig. 2A, human primary skeletal muscle myotubes from obese participants treated with high C3G (100 μM), low and high peptide concentrations as well as their combinations exhibited significantly lower AGTR-1 expression compared to control treatment ($P < 0.05$, n = 8). The similar results were also observed in the obese T2DM group. AGTR-1 expression was significantly reduced ($P < 0.05$), in myotubes from obese T2DM participants following the treatment with both low and high peptide concentrations and the combination of high peptide (1500 μg/mL) with low C3G concentration(10 μM) for 24 h (n = 9, Fig. 2B). However, high C3G concentration alone and its combination with peptides did not alter AGTR-1 expression in human primary myotubes obtained from obese T2DM participants, compared with control treatment (Fig. 2B).

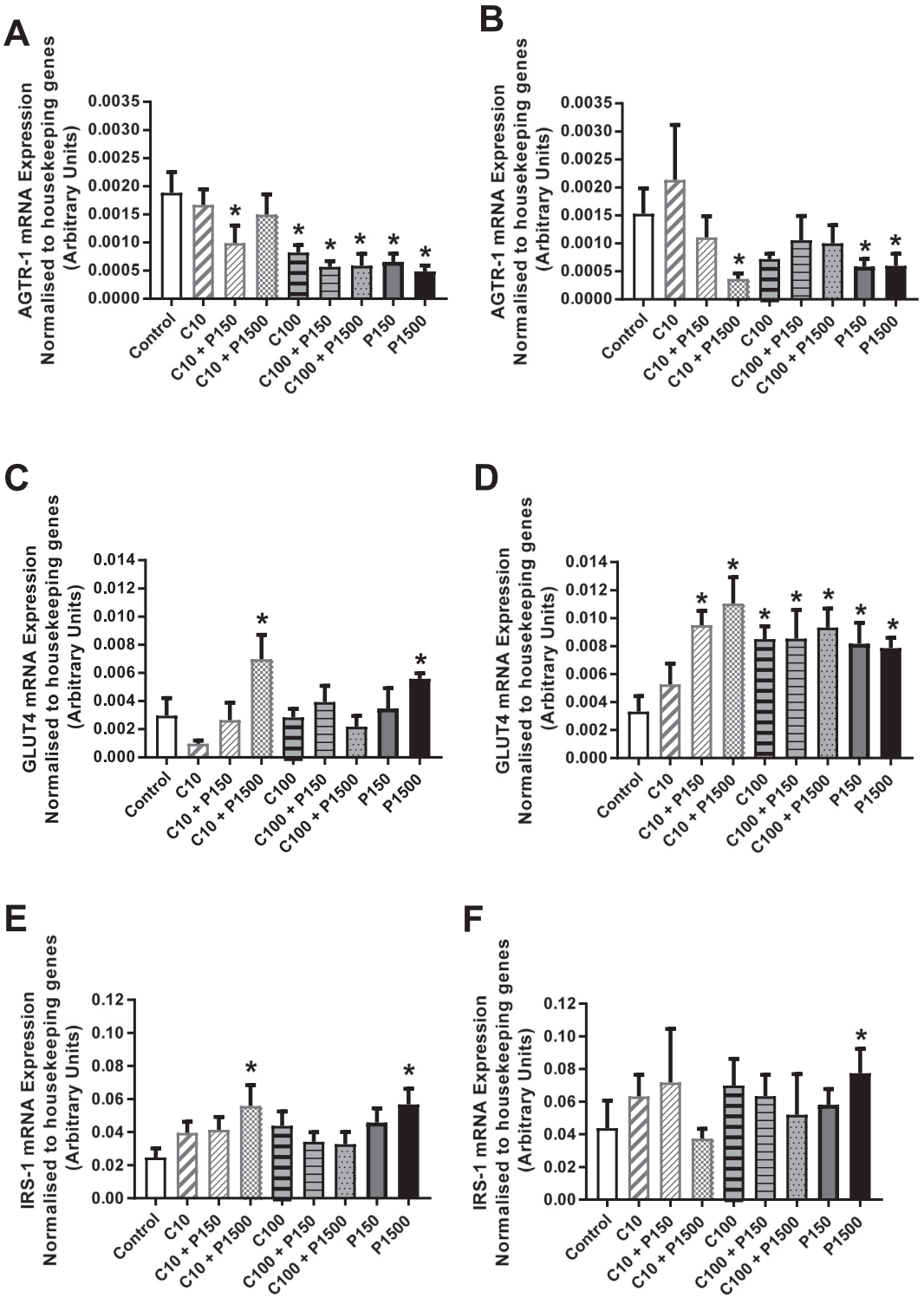

Fig. 2. The expression of AGTR-1, GLUT4, IRS-1 and FOXO1 in human primary skeletal muscle myotubes derived from obese and obese T2DM participants. Cells were treated with 10 μM and 100 μM of C3G, 150 μg/mL and 1500 μg/mL of peptides, and combinations of C3G/peptides for 24 h. All genes were normalised to the average of two housekeeping genes, GAPDH and cyclophilin. Data was expressed as mean ± SEM (n = 8–10). Significance is indicated by * $P < 0.05$. The expression of (A) AGTR-1, (C) GLUT4, (E) IRS-1 and (G) FOXO1 in human primary skeletal muscle myotubes derived from obese participants. The expression of (B) AGTR-1, (D) GLUT4, (F) IRS-1 and (H) FOXO1 in human primary skeletal muscle myotubes derived from obese T2DM participants.

1500 μg/mL of peptide combined with 10 μM of C3G up-regulated the mRNA expression of GLUT4 in the obese group (Fig. 2C). For the obese T2DM group, high C3G, high and low peptide concentrations alone, as well as their combinations significantly increased the mRNA expression of GLUT4. However, low C3G concentration (10 μM) did not change the expression of this gene (Fig. 2D). GLUT4 mRNA was significantly up-regulated when 10 μM of C3G was combined with 150 μg/mL ($P = 0.011$, n = 10) and 1500 μg/mL of peptide ($P = 0.008$, n = 10), compared with low C3G concentration (10 μM) alone in the obese T2DM group (Fig. 2D).

The mRNA expression of IRS-1 was significantly increased in myotubes derived from obese participants following treatments with high

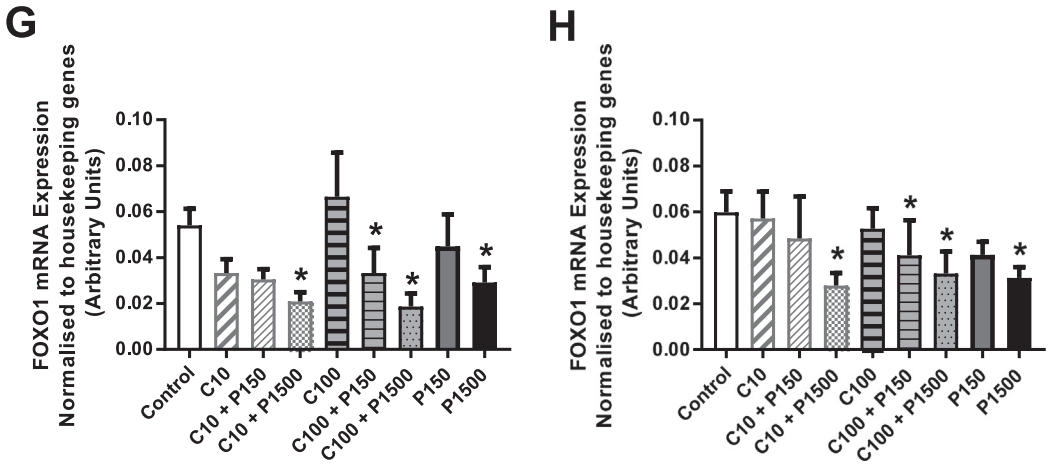

Fig. 2. (*continued*)

peptide concentration alone as well as combined with low C3G concentration ($P < 0.05$, n = 10, Fig. 2E). However, in the obese T2DM group, only high peptide treatment increased the expression of IRS-1 compared with control treatment ($P = 0.016$, n = 10, Fig. 2F).

Low C3G and peptide concentrations alone as well as in combination did not alter FOXO1 mRNA expression significantly in human skeletal muscle myotubes. A similar result was also observed following the treatment of high C3G concentration (Fig. 2G, H). In contrast, high peptide concentration alone or in combination with both low and high C3G concentrations ($P < 0.05$, n = 10) resulted in a significant reduction in the mRNA expression of FOXO1 in both obese and obese T2DM groups, compared with the control treatment.

3.3. 'Real-time' PCR analysis of the expression of others genes related to glucose metabolism in human primary skeletal muscle myotubes obtained from obese and obese T2DM participants

Fig. 3 A showed that GLUT1 mRNA expression was up-regulated by the high peptide concentration (1500 μg/mL) in the obese group, compared with the control treatment. Treatments with C3G or peptides alone did not alter GLUT1 mRNA expression significantly in human skeletal muscle myotubes obtained from obese T2DM participants (Fig. 3B). In contrast, the combination of 100 μM of C3G and 150 μg/mL of peptide ($P = 0.012$, n = 8, Fig. 3B) significantly increased the expression of GLUT1 mRNA in myotubes from T2DM participants, compared with the control treatment.

C3G or peptides alone did not alter IRS-2 mRNA expression in the obese group (Fig. 3C) or obese T2DM group (Fig. 3D), however it was down-regulated in both groups by the combinations of high C3G concentration with both low and high doses of peptides (150 μg/mL and 15000 μg/mL; $P < 0.05$, Fig. 3C, D).

All treatments including low and high C3G concentrations and their combinations with low and high peptide concentrations ($P < 0.05$, Fig. 3E), but not high peptide concentration alone, significantly reduced the mRNA expression of PI3K in the obese group. High C3G concentration and its combinations with peptides (both of low and high concentrations) resulted in a significant reduction in mRNA expression of PI3K in the obese T2DM group, and the combination of low C3G (10 μM) and high peptide concentration (1500 μg/mL) ($P < 0.05$, Fig. 3F). The other treatments did not alter mRNA expression of PI3K in the obese T2DM group.

There was no change in the mRNA expression of AMPK following the treatments of C3G and peptides alone, as well as the combinations of low C3G concentration with peptides in the human primary skeletal muscle myotubes from obese participants. But peptides combined with high C3G concentration resulted in a down-regulation in the mRNA expression of AMPK in the same group (Fig. 3G). On the other hand, no treatment caused significant changes in the mRNA expression of AMPK, compared with control in the obese T2DM group (Fig. 3H).

C3G and peptide treatments alone and their combinations did not alter the mRNA expression of PPAR-α in the obese group (Fig. 3I). However, high C3G concentration (100 μM) alone and its combination with peptides (both low and high concentrations) ($P < 0.05$, Fig. 3I) down-regulated the mRNA expression of PPAR-α, compared with the control treatment. In the obese T2DM group, the mRNA of PPAR-α was not significantly different following the treatments of low C3G alone or peptides alone (both concentrations, Fig. 3J). In contrast, the expression of PPAR-α was decreased with the treatments of high peptide concentration combined with both concentrations of C3G, as well as high C3G alone ($P < 0.05$, Fig. 3J).

4. Discussion

Anthocyanins extracted from plants have previously been shown to increase glucose uptake in both L6 and C_2C_{12} myotubes as well as in 3T3-L1 adipocytes (Harbilas et al., 2009; Martineau et al., 2006; Rojo et al., 2012). ACE inhibitors have also been found to enhance the whole-body glucose disposal and glucose transport activity in the skeletal muscle, in a variety of insulin-resistant animal models (Henriksen, et al., 2001; Sugiishi et al., 2013) and insulin-resistant humans with essential hypertension (Kinoshita et al., 2002). The present study is the first to look at the synergistic effects of C3G and peptides with ACE inhibitory activity on glucose uptake in human primary myotubes derived from obese and obese T2DM participants.

In this study, high concentration of C3G in the presence of insulin significantly increased glucose uptake, compared with insulin treatment alone in the obese group, but low C3G concentration in the presence or absence of insulin did not change glucose uptake in either the obese or obese T2DM groups. In agreement with our result, it has previously been demonstrated that an increase in glucose uptake of L6 myotubes was obtained with higher concentrations of anthocyanin combined with insulin (Rojo et al., 2012). It is well known that human primary myotubes express obese or diabetic phenotypes when they are established from participants with obesity or diabetes (Berggren, Tanner and Houmard, 2007; Thingholm, Bak, Beck-Nielsen, Jensen and Gaster, 2011). A previous study showed that a reduction of glucose transport stimulated by insulin was observed in myotubes only from obese T2DM participants, not from nondiabetic obese participants, indicating that differentiated myotubes raised from patients with T2DM remain insulin resistant *in vitro* (Henry, Abrams, Nikoulina and Ciaraldi, 1995). In this current study, only donors who had diabetes demonstrated the characteristics of diabetes with elevated plasma insulin levels and high fasting blood glucose, while donors who were obese but not diabetic did not display any evidence of hyperglycaemia (Table 1).

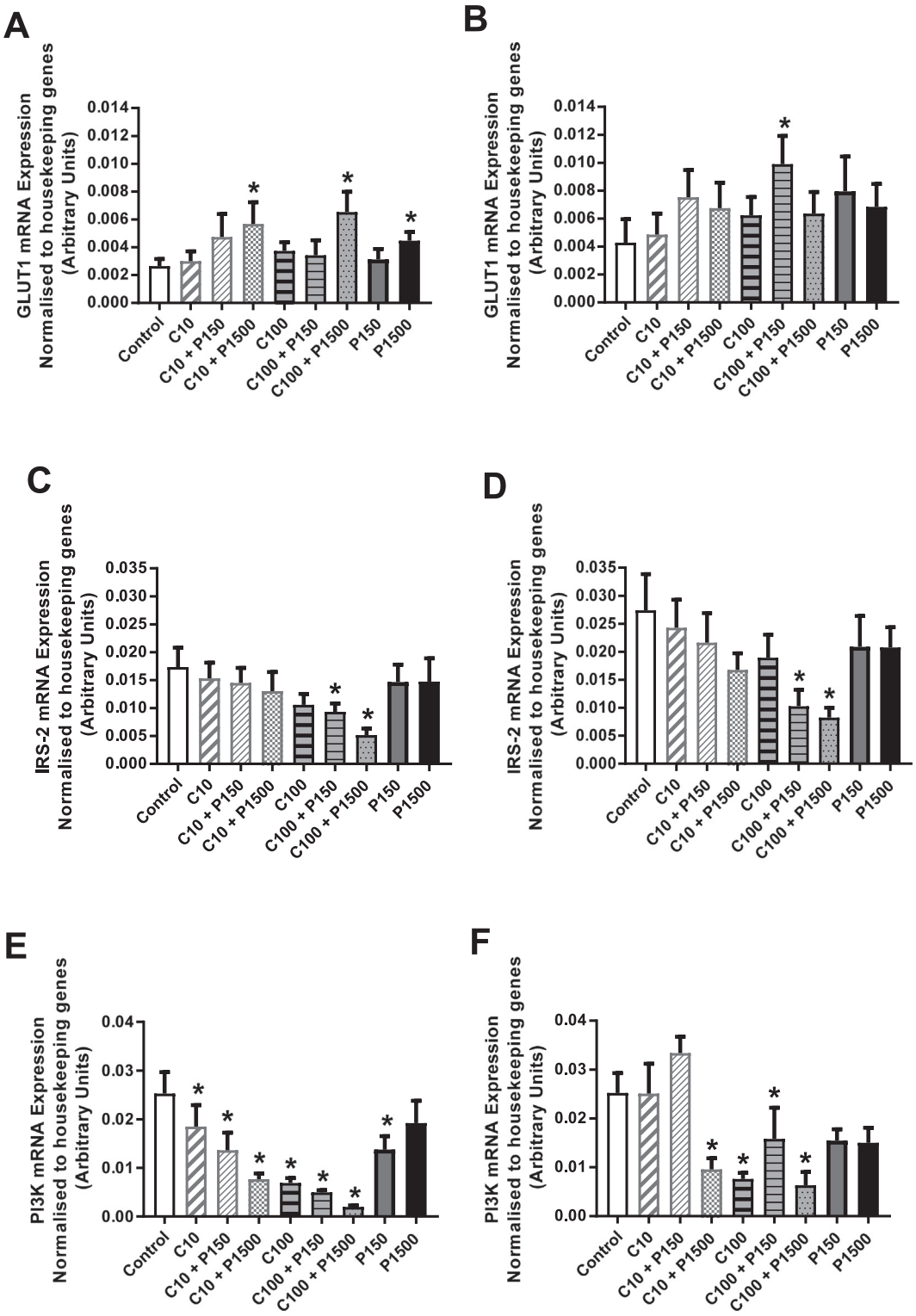

Fig. 3. The expression of GLUT1, IRS-2, PI3K, AMPK and PPAR-α in human primary skeletal muscle myotubes derived from obese and obese T2DM participants. Cells were treated with 10 μM and 100 μM of C3G, 150 μg/mL and 1500 μg/mL of peptides, and combinations of C3G/ peptides for 24 h. All genes were normalised to the average of two housekeeping genes, GAPDH and cyclophilin. Data are expressed as mean ± SEM. Significance is indicated by * $P < 0.05$ compared to control treatment (n = 8–10). The expression of (A) GLUT1, (C) IRS-2, (E) PI3K, (G) AMPK and (I) PPAR-α in human primary skeletal muscle myotubes derived from obese participants. The expression of (B) GLUT1, (D) IRS-2, (F) PI3K, (H) AMPK and (J) PPAR-α in human primary skeletal muscle myotubes derived from obese T2DM participants.

Therefore, differential effects of the treatments on glucose uptake between obese and obese T2DM groups were possibly associated with human primary myotubes preserving their phenotype. In addition, the variation of fibre types, mitochondrial function and mitochondrial capacity in myotubes from different groups may have also exacerbated the differences following the treatments (Minet and Gaster, 2010; Thingholm, et al., 2011). The differences observed in the current study between obese and T2DM cell lines are however consistent with

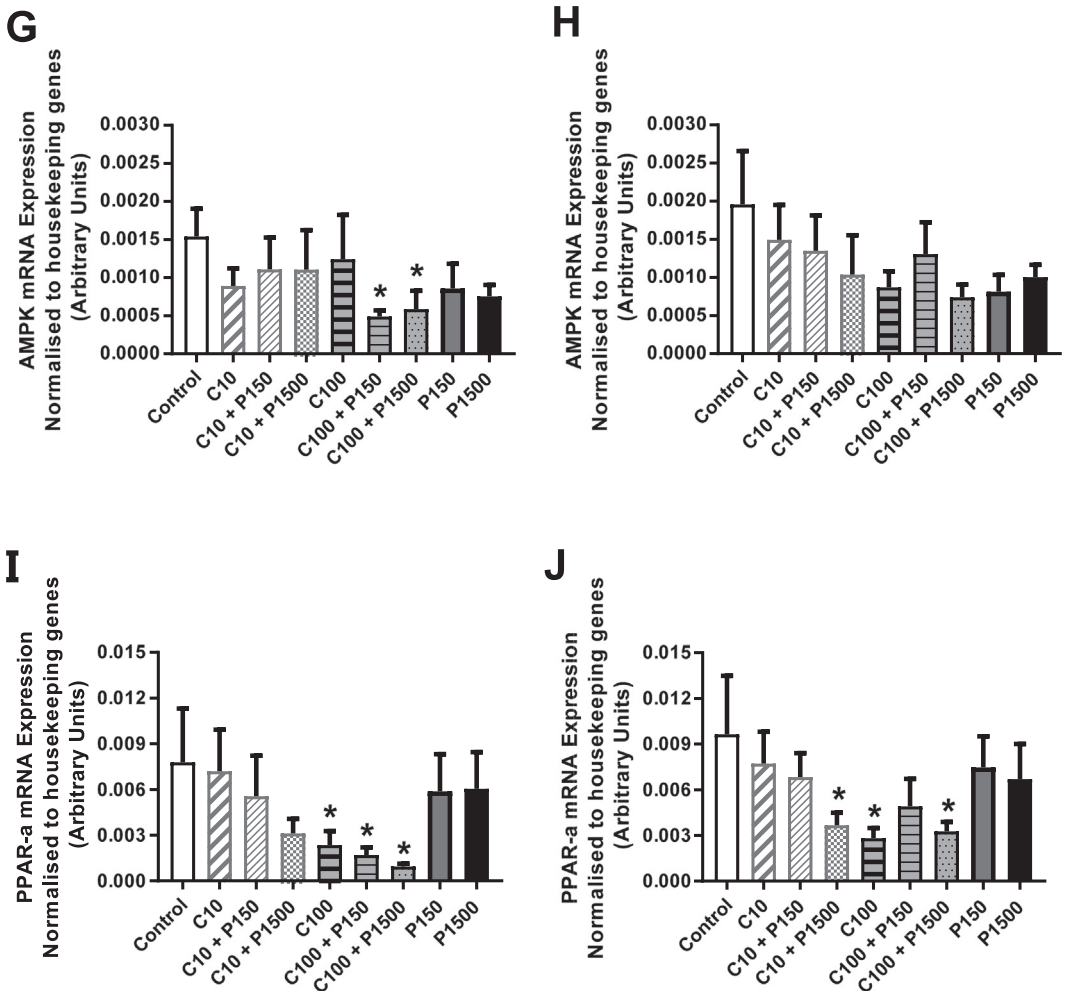

Fig. 3. (*continued*)

previous studies, such as those reported with globular adiponectin with variations in a dose effect on AMPK activity in human myotubes obtained from both obese and obese T2DM participants (Chen et al., 2005). This finding also suggests that peptides with ACE inhibitory activity have a potential to improve insulin sensitivity and attenuate insulin resistance in both obese and obese T2DM patients, however this may be dose dependent in the obese T2DM state. It has been reported that ACE inhibitors on skeletal muscle glucose uptake were associated with insulin signalling up-regulation, including enhanced IRS-1 tyrosine phosphorylation and PI3K activity (Dal Ponte, Fogt, Jacob and Henriksen, 1998; Henriksen and Jacob, 2003), which was consistent with our findings that high peptide concentration improved the expression of IRS-1 in human primary myotubes in both obese and obese T2DM groups. *In vivo* studies have reported that the ACE inhibitors captopril (Dal Ponte, et al., 1998) trandolapril (Steen et al., 1999) or imidapril (Nawano, et al., 1999) could improve oral glucose tolerance and whole-body insulin sensitivity in obese Zucker rats. In this study, peptides with ACE inhibitory activity were extracted from yoghurt fermented by *L. helveticus*. It has been reported that *L. helveticus*, one of the most efficient lactic acid bacteria, possess an efficient proteolytic system capable of producing short peptides from the casein matrix (Sadat-Mekmene et al., 2011). Furthermore, the small size peptides that were produced, contained proline and hydroxyproline residues, and can usually resist breakdown by digestive enzymes, thus they can arrive to intestine due to detection in faecal samples after ingestion (Saito, Sakamoto, Takizawa and Benno, 2004; Seppo, Jauhiainen, Poussa and Korpela, 2003). Therefore, these small peptides that exerted the regulatory function observed on glucose metabolism in the current study may be able to exert their function *in vivo* due to their bioavailability.

To further explore the mechanisms underlying the efficacy of C3G and peptides with ACE inhibitory activity in promoting glucose uptake in human primary myotubes, multiple genes involved in glucose metabolism were assessed. To the best of our knowledge, this is the first study to explore the mRNA expression of AGTR-1 in human primary myotubes following the treatments of C3G and/or peptides that have ACE inhibitory activity. Based on our findings that C3G and/or peptide treatments significantly down-regulated mRNA expression of AGTR-1, and significantly increased the mRNA expression of IRS-1, in both obese and obese T2DM groups, may suggest a possible crosstalk between Ang II and insulin-signalling pathways. Ang II induces insulin resistance via tyrosine phosphorylation of IRS-1 associated with the AGTR-1, thereby attenuating insulin-induced activation of PI3K associated with IRS-1 (Dal Ponte et al., 1998; Folli, Kahn, Hansen, Bouchie, & Feener, 1997; Fujimoto et al., 2004; Ogihara et al., 2002). ACE inhibitors also up-regulate the mRNA expression of GLUT4 in skeletal muscle (Carnagarin, Dharmarajan and Dass, 2015). Consistent with these observations our results also revealed that the expression of GLUT4 was significantly increased by all treatments except low C3G concentration alone in the obese T2DM group, although the increase in mRNA expression, was not translated into an increase in glucose uptake with the exception of the high peptide concentration in isolation. The mechanisms by which C3G and/ or peptides improved glucose uptake in human primary myotubes remains unclear, however a down-regulation of AGTR-1 mRNA and an up-regulation of GLUT4 mRNA expression may indicate that these bioactive components have a role in glucose metabolism, but further analysis is required.

FOXO1, which belongs to the Forkhead family of transcription factors, has a role in skeletal muscle insulin and glucose homeostasis. The over expression of FOXO1 has been shown to impair glycaemic control as a result of reduced skeletal muscle mass (Sugiishi et al., 2013). The activated FOXO1, as a negative regulator of insulin sensitivity, plays a critical role in muscle insulin resistance (Wang et al., 2017). Previous studies have reported that insulin dramatically reduced the FOXO1 nuclear level in insulin-stimulated vehicle-treated myotubes (Southgate et al., 2005), significantly decreasing the stability of FOXO1 protein (Matsuzaki, Daitoku, Hatta, Tanaka and Fukamizu, 2003), as well as suppressing FOXO1 transcriptional activity. A recent study has shown that the reduction in FOXO1 could increase GLUT4 expression in transfected C_2C_{12} cells with siRNA-FOXO1 (Wang et al., 2017). In the present study, mRNA expression of FOXO1 was suppressed by high peptide concentration and its combination with C3G in both obese and obese T2DM groups, suggesting that this inhibition may be attributed to, at least partially, to peptides and C3G having insulin-like properties. Further analysis however, is required to elucidate the mechanisms that caused this observation in human primary myotubes.

It has been established that activation of AMPK increases glucose uptake in skeletal muscle and helps regulate blood glucose homeostasis (Naimi et al., 2017). In the current study, the expression of AMPK was decreased with the treatments of high C3G combined with both low and high peptide concentrations in the obese group, while no change was observed in the obese T2DM group. These results are in conflict with previous published findings in which fermented blueberry juice (that had *Serratia vaccinii* bacterium added to it) increased glucose uptake by 48% in C_2C_{12} myotubes as a result of AMPK activation (Vuong, Martineau, Ramassamy, Matar and Haddad, 2007). It has also been demonstrated that dietary bilberry extract significantly activated AMPK in skeletal muscle, and enhanced glucose uptake into skeletal muscle tissue (Takikawa, Inoue, Horio and Tsuda, 2010). The different experimental design, such as species (human primary *vs* mouse immortalised cell-line), phenotype of myotubes (human primary myotubes obtained from obese and T2DM participants *vs* C_2C_{12} myotubes), the treatments (single compound *vs* mixture including various anthocyanins and experimental system (*in vivo vs in vitro*) could account for the differences observed between previously reported data and the current study.

In this study, only mRNA of AMPK and GLUT4 were measured, but future determination of phosphorylation of AMPK and the translocation of GLUT4 would be of value to ensure the changes in mRNA were translated into functional changes within the cell. Numerous studies however, have shown that mRNA of AMPK and GLUT4 are correlated with phosphorylation of AMPK and the translocation of GLUT4 (Chen, Lin and Shih, 2014; Dhanya, Arya, Nisha and Jayamurthy, 2017; Han et al., 2015; Huang et al., 2010). Thus mRNA expression of AMPK and GLUT4 still can be recognized as suitable markers to assess glucose metabolism in human primary myotubes.

5. Conclusions

In summary, the results presented here demonstrate that high C3G concentration in combination with peptides with high ACE inhibitory activity extracted from yoghurt (both low and high concentrations) can enhance glucose uptake with or without insulin stimulation in human primary myotubes from obese participants. Only high peptide concentration however, increased glucose uptake in myotubes derived from obese T2DM participants in the absence of insulin. It is possible that the peptides extracted from yoghurt with high ACE inhibitory activity increased glucose uptake through the inhibition of AGTR-1 mRNA expression, and subsequently the increase of IRS-1 and GLUT4 mRNA expression. C3G also demonstrated its potentially anti-diabetic activity, at least in part, in an insulin-dependent signalling pathway, associated with the increase of IRS-1 mRNA expression and GLUT4 mRNA expression. However, the combinations of C3G and peptides appear to have not had an additive effect on either glucose uptake or the regulation of multiple genes. The results suggest that C3G and peptides with ACE inhibitory activity provide a novel insight into the potential implications of natural anti-diabetic compounds for the regulation of glucose metabolism in obese and obese T2DM participants. Further research is required to elucidate the mechanisms of these active compounds involved in the signalling pathways via regulation of the related protein expression and translocation of GLUT4 to the cell membrane. Moreover, the efficacy of C3G and peptides on glucose metabolism will need to be validated through human clinical trials.

6. Ethics statement

This original manuscript obtained ethical approval for this study from The Human Ethics Research Committee of Victoria University, Melbourne Australia (HRE08-158) and all participants gave written informed consent.

7. Author contributions

MS, XQS and AJM conceived and designed the experiments. MS conducted experiment, collected data and drafted the manuscript. XQS, AJM, LOK and AS critically reviewed manuscript. AJM, LOK and AS collected and established the cell lines. All authors read and approved the manuscript.

Conflict of interest

The authors have no conflict of interest to declare.

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