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Di and tripeptides from marine sources can target adipogenic process and contribute to decrease adipocyte number and functions

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Abstract

The effect of 11 marine-derived cryptides was investigated on proliferation, differentiation and maturation of human white pre-adipocytes (HWP). They were all formerly identified as potent Angiotensin-Converting-Enzyme inhibitors. Val-Trp (VW), Val-Tyr (VY), Lys-Tyr (KY), Lys-Trp (KW), Ile-Tyr (IY), Ala-Pro (AP), Val-Ile-Tyr (VIY), Leu-Lys-Pro (LKP), Gly-Pro-Leu (GPL), Ala-Lys-Lys (AKK) and Val-Ala-Pro (VAP) were previously found in fish products and co-products as well as other marine resources like wakame. Treatment with AP, VAP and AKK greatly affected viability of HWP during the proliferation period while KW and VW treatment reduced the number of viable cells during the differentiation stage. A GPL and IY incubation during the differentiation stage allowed the decrease of their final lipid content, of the GPDH activity and of the mRNA level of adipocytes markers (aP2, GLUT4, LPL and AGT). Moreover, a down regulation of both PPARγ and C/EBPα expression, two key regulators of adipogenesis was observed. These findings indicate that small bioactive peptides from marine protein hydrolysates can target adipogenesis and thus could regulate energy metabolism disorders.

Keywords: Bioactive peptides, Obesity, Human white pre-adipocytes, proliferation, differentiation, adipocyte differentiation markers.

1. Introduction

Around 1.6 billion adults (over the age of 15 years) are considered to be over weighted (with a body mass index (BMI) between 25 and 30 kg/m^2) and 400 million are considered obese (with a BMI $\geq$ 30 kg/m^2) (Stothard, Tennant, Bell, & Rankin, 2009). These data demonstrate the urgency to establish effective strategies for prevention and treatment of obesity. Among ways to reduce obesity, the decrease of energy/food intake and the increase of energy expenditure (Park, Jeon, Kim, & Han, 2013) are of importance but targeting adipocyte physiology and cell cycle could also be a solution. Indeed, recent studies have examined how far inhibition of preadipocyte proliferation, differentiation, lipogenesis or promotion of lipolysis and fat oxidation (Wang, & Jones, 2004) can reduce obesity. Obesity is characterized at the cellular level by an increase in the number and size of adipocytes originating from fibroblastic pre-adipocytes in adipose tissues (Furuyashiki et al., 2004). Pre-adipocytes, which can then be differentiated into mature adipocytes and modulate body fat mass, play a key role in obesity (Park et al., 2013).

At a molecular level, differentiation is a well-organized program characterized by the loss of pre-adipocyte markers and sequential changes in the expression of general and adipocyte-specific genes that determine the specific adipocyte phenotype (Catalioto, Maggi, & Giuliani, 2009). These changes in gene expression during differentiation program are induced by a coordinated expression and action of transcription factors. Consequently, early, intermediate and late mRNA and protein markers can be detected and finally triglycerides (TG) accumulation can be observed.

Moreover, mature adipocytes express all components of the renin-angiotensin system (RAS), including angiotensinogen (AGT), the sole precursor of Angiotensin II (Ang II), type 1 (AT1) and type 2 (AT2) angiotensin receptors as well as the angiotensin peptide forming enzymes: renin, angiotensin-I-converting enzyme (ACE), and chymase (Engeli, Negrel & Sharma,

2000). This local RAS exerts important auto/paracrine functions in modulating lipogenesis, lipolysis and adipogenesis (Massiera et al., 2001). Ang II, a potent vasoconstrictor and key player in hypertension, increases lipogenesis and TG accumulation in 3T3-L1 pre-adipocytes and human adipocytes (Jones, Standridge, & Moustaid, 1997). In addition, Crandall et al. (1999) observed that the stimulation of human pre-adipocytes with Ang II resulted in an acceleration of the G1-phase of the cell cycle and an increased expression of the cell cycle regulator *cyclin D1*. This suggests that Ang II can contribute to the growth and the development of adipose tissues. Thus blockade of the RAS and its main enzyme, ACE, may be of particular benefit in the management of obesity probably by reducing adipose size and decreasing functional adipocytes number. ACE- inhibitors (ACEI) may thus contribute to attenuate obesity by acting on adipocyte life cycle.

Since synthetic ACE inhibitors have often side effects, dietary bioactive peptides able to inhibit ACE could be interesting alternatives in the management of obesity.

We previously demonstrated that incubation of human adipocytes with a goat whey hydrolysate containing ACEI peptides decreased the proliferation of pre-adipocyte and reduced the lipid content of mature adipocytes (Hammé, Sannier, Piot, & Bordenave-Juchereau, 2010). The aim of this current work is to assess whether cryptides, bioactive peptides hidden within the sequence of a parent protein, already identified as potent ACEI can act on adipogenesis. These cryptides were selected in marine products such as fish, seaweed, and shellfish for their previously high inhibitory ACE potency, as demonstrated in administration studies with spontaneously hypertensive rats (SHR) (Ono, Hosokawa, Miyashita, & Takahashi, 2003). They also were chosen for their shortness giving them the opportunity to penetrate in tissue or cells and to resist to endogenous proteases. They can be found in sea bream, sardine or chicken indicating that theses sequences are ubiquitous.

We investigated the ability of 6 dipeptides (Val-Trp (VW), Val-Tyr (VY), Lys-Tyr (KY), Lys-Trp (KW), Ile-Tyr (IY), Ala-Pro (AP)) and 5 tripeptides (Val-Ile-Tyr (VIY), Leu-Lys-Pro (LKP), Gly-Pro-Leu (GPL), Ala-Lys-Lys (AKK), Val-Ala-Pro (VAP)) to act on adipocyte development. A quite high concentration of 100µM was chosen for all cryptides tested since it exhibited a noticeable effect on adipocytes development. Viability was measured after proliferation and differentiation of adipocytes. The regulatory effect of these peptides on the differentiation stage was analyzed by measuring the mRNA expression changes of adipocyte differentiation markers as well as assessing GPDH activity. Effect of peptides on lipid accumulation was also monitored after various times of exposition.

2. Material and methods

2.1. Material

Human white pre-adipocyte (HWP) were purchased as cryopreserved pre-adipocytes from PromoCell (Heidelberg, Germany) and originated from subcutaneous white adipose tissue obtained from female/62 years/Caucasian. The growth and differentiation media for pre-adipocytes, the nutrition medium for adipocytes and supplements for each medium were purchased from PromoCell.

The antibiotic solution containing Penicillin 10,000 U / ml and Streptomycin, 10 mg / ml was from PAN Biotech. GmbH (Aidenbach, Germany).

MTT (3-(4.5-dimethylthiazolyl)-2.5-diphenyl-tetrazoliumbromide), DMSO, Caffeine, Oil red O stain and Bradford reagent were from Sigma–Aldrich (Saint-Quentin Fallavier, France).

Synthetic peptides were purchased at >90% of purity from GeneCust (Dudelange, Luxembourg). Purity of each peptide was checked after solubilisation in adequate buffer and their molecular mass and aminoacid sequence were confirmed by HPLC ESI-MS.

2.2. Cell Culture

HWP cells were cultured according to the manufacturer's instructions with slight modifications. Briefly, cells not exceeding 4 passages were cultured at 37 °C in a humidified 5% CO_2 atmosphere and grown in a preadipocyte Growth Medium (GM) with 1% antibiotics (proliferation stage).

For adipocyte differentiation, confluent cultures (Day 0) were stimulated to differentiation with pre-adipocyte differentiation medium (DM) containing d-Biotin 8 µg/ml, insulin 0.5 µg/ml, dexamethasone 400 ng/ml, isobutylmethylxanthine 44 µg/ml, L-thyroxine 9 ng/ml and

ciglitazone 3µg/ml. After 4 days, medium was changed to the adipocyte nutrition medium (NM) containing Fetal Calf Serum 3%, d-Biotin 8µg/ml, insulin 0.5 µg/ml and dexamethasone 400 ng/ml, Cells were cultured in nutrition medium for further 4, 8 or 12 days according the test. By day 16, cells contained large lipid droplets and were considered as mature adipocytes.

Cryptides, dissolved in dimethyl sulfoxide (stock solution, 10^{-2}M), were added at a final concentration of 100 µM to GM or DM and/or NM in order to observe their effects. Each medium was replaced every 2 days by fresh medium containing cryptides.

2.3. MTT assay

To detect the effect of marine cryptides on the viability of HWP during proliferation stage, HWP were seeded in 96-well culture plates at a density of 5000 cells/well and cultured in GM with or without 100 µM of marine cryptides. After 24 h or 72 h of treatment, 25µl MTT (5mg/mL) were added to each well. The plates were incubated at 37°C for 4 h, followed by the addition of DMSO (200 µl/well), and incubated at 37°C for 10 min. Optical density (OD) was measured at 570 nm using UV spectrometer Devices versa Max microplate reader (Molecular devices, California, United States). Non treated-cells were used as control.

To detect the effect of some cryptides on the viability of HWP cells during differentiation stage, cells were cultured with 100 µM of marine cryptides from day 0 to day 12. MTT assay was carried out on days 3, 6, 9 and 12 using the method described above.

2.4. Oil-Red-O staining and quantification of lipid accumulation

Lipid accumulation was estimated in mature adipocytes, cultured in 24-well plates at a density of 10^4cells/ml. On day 16, medium was removed and cells were washed twice with PBS, fixed 1h with 500 µl 4% formaldehyde in PBS at room temperature, rinsed with 500 µl 60%

2-propanol and dried for 5min. Cells were stained with 200 µl Oil-Red-O solution (Oil-Red-O stock solution (5mg/ml in pure isopropanol): water=3:2)) at room temperature for 10 min. Then the Oil-Red-O solution was removed and cells were washed 4 times with distilled water to remove excess of stain. Images were obtained using Eclipse TS100 inverted microscope (100X) equipped for phase-contrast microscopy (Nikon, Yokohama, Japan).

For quantification, the stained oil droplets were dissolved in 750 µl isopropanol for 10 min and the lipid content evaluated by measuring the optical absorbance at 540 nm using Fluostar Omega spectrofluorometer (BMG LABTECH GmbH, Ortenberg, Germany).

2.5. Quantification of the GPDH activity

HWP were seeded in 24-well plates, grown to confluence and treated during the differentiation period (from day 0 to day 8) with 100µM of cryptides (treated cells). A control with medium only was included in the study. On day 8, cells were washed twice with PBS, then enzyme extraction buffer was added to induce cell lysis, and cells extracts were collected by scraping. After centrifugation at 10,000 rpm for 5 min at 4°C, the glycerol-3-phosphate dehydrogenase (GPDH) specific activity of supernatant was measured with the GPDH Activity Assay Kit (TaKaRa Bio Inc., Shiga, Japan). GPDH Protein concentration was measured using the Bradford protein assay (Bradford, 1976). Values for GPDH activity were expressed as a proportion of control (100%).

2.6. Real time PCR

HWP were seeded at 10^5 cell /ml in six well plates, grown to confluence, and then treated with 100µM of cryptides during differentiation period (from day 0 to day 8). A control with medium only was included in the study. On day 8, cells were collected. Total RNA was extracted using the NucleoSpin RNA II kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. The RNA samples were treated using the Ambion DNA-free

kit (Life Technologies, Carlsbad, USA). RNA was quantified at 260 nm using NanoDrop 2000 UV-Vis Spectrophotometer (Thermo Scientific, USA), and sample integrity was checked on 1% agarose gel electrophoresis.

Reverse-transcription of RNA (500 ng) was carried out using the Cloned AMV First-Strand cDNA Synthesis Kit (Invitrogen, Carlsbad, USA), with random primers following the manufacturer's instructions.

A real time PCR was then performed on cDNA samples to evaluate the mRNA expression of human genes PPARγ, C/EBPα, aP2, GLUT4, LPL and AGT. Human β-actin was used as an endogenous reference.

Reactions were run on an Mx3000P Real-Time Thermocycler (Stratagene, La Jolla, CA,USA) using Brilliant III Ultra-Fast SYBR Green kit (Applied Biosystems, Foster City, CA, USA) with the following cycling parameters: 1 cycle at 95°C for 3 min followed by 40 cycles at 95°C for 10s and 60°C for 20 s. Each reaction was performed in duplicate in a final volume of 20µl containing 5µL cDNA (1/20 dilution), 2× Brilliant III Ultra-Fast SYBR Green Master Mix (10µL), and with an optimal concentration of gene specific forward and reverse primers (400 nM). The primer (Eurofins MWG Operon, Ebersberg, Germany) sequences are presented in table 1.

Relative quantification values were expressed using the $2^{-\Delta\Delta Ct}$ method: the amount of target message (PPARγ, C/EBPα, aP2, GLUT4, LPL, or AGT in mRNA treated adipocytes) was normalized to the internal reference (β-actin) and compared to the calibrator (PPARγ, C/EBPα, aP2, GLUT4, LPL, or AGT in untreated adipocytes).

The primers were validated by testing PCR efficiency using standard curves (95%≤ efficiency ≤105%). PCR product specificity was evaluated by generating a dissociation curve following

194 the manufacturer's recommendations. All results were obtained from at least three
195 independent experiments.

196 ***2.7. Statistical Analysis.***

197 Data are presented as means ± standard error. Statistical analyses were conducted using
198 software Origin 6.0. A significant difference from the control group was analyzed with an
199 unpaired Student *t* test. *P* values <0.05 data were considered significantly different from those
200 of the control.

201

202 **3. Results**

203 ***3.1. Effect of marine cryptides on the viability of HWP cells during proliferation and***
204 ***differentiation***

205 To detect the effect of marine cryptides on viability of HWP during the proliferation stage,
206 cells were treated with 100 μM of VW, KW, IY, KY, VIY, VY, LKP, GPL, AKK, AP, and
207 VAP. MTT analysis was carried out after 24 h (Figure 1A) and 72 h (Figure 1B) of treatment.
208 As shown in Figure1A; AP, VAP and AKK affected cell viability significantly (p<0.01). VAP
209 exhibited the greatest cell viability inhibition with a decrease of viability of 59.3±7.3% and
210 81.1±4.3% after 1 and 3 days of treatment respectively when compared to controls. AP and
211 AKK decreased viability by 46.4±3.9% and 24.5±5.3% and by 55.8±4.5 % and 22.8 ±4.3%,
212 respectively, after 1 and 3 days of incubation while other cryptides tested showed no
213 significant effect (p>0.05) on the HWP viability after 1 and 3 days of treatment during the
214 proliferation stage. After 24 h, differences among effective peptides was significant between
215 AKK and AP, VAP and AP, but not between AKK and AP. After 72 h, all values obtained
216 after treatment with AKK, AP and VAP were significantly different.

Peptides exhibiting no anti-proliferative action during growth stage were then added in HWP medium during the differentiation period. 100 µM of VW, VY, IY, KY, VIY, KW, GPL and LKP were added on cells and MTT analysis was carried out after 3, 6, 9 and 12 days during the differentiation stage. The cell viability was not affected after 72 h except in the presence of VW and KW which were found to reduce significantly ($p<0.05$) the number of viable cells when compared with control (Figure 1C). For example, after 12 days, VW and KW decreased cell viability by 32.8±5.1% ($p<0.05$) and 26.8±4.3% ($p<0.05$) respectively.

3.2. Effect of marine cryptides on residual lipid content of mature adipocytes

The inhibitory effect of cryptides on the final lipid content of mature adipocytes was studied, using those which did not exhibit adverse effect on adipocyte viability (GPL, IY, VIY, KY, VY and LKP). 100 µM of cryptides or 100 µM caffeine (chosen as a lipolytic reference molecule) was added to the culture medium. Figure 2A, B, C, D show the staining observed and Fig. 3, the quantitative results of mature adipocytes at day 16. As expected, the staining of HWP cultured in DM and NM without treatment revealed plenty of lipid droplets, indicating an almost total differentiation (Figure 2A).

Cells grown in DM and NM in presence of GPL (Figure 2B), IY (Figure 2C) or caffeine (Figure 2D), showed less oil droplets than the control cells indicating an inhibition of lipid filling when cells were treated with these cryptides. GPL treatment showed the maximum reduction on lipid content compared with control group (Figure 2A). This was confirmed by the quantitative Oil-Red-O staining measurements (Figure 3), which indicated that HWP grown in the presence of GPL exhibited a reduction of the lipid content by 20.9±4.0 % ($p<0.05$). In addition, IY and VIY reduced significantly ($p<0.05$) the final lipid accumulation by 10.4±4.1 % and 9.3±2.5 %, respectively.

Analysis of the results obtained with KY, VY and LKP (Figure 3) showed no significant difference ($p > 0.05$) when compared with control cells.

As we observed a reduction of the final lipid content after adipocytes incubation with GPL, IY, and VIY during the differentiation stage, we then examined if these peptides could potentially act on human adipose conversion at early or late stages of the differentiation programs.

For that purpose, 100 µM of IY, GPL, and VIY were added to the differentiation medium or to the nutrition medium. The extent of differentiation process leading to the filling of adipocytes with lipid droplets was monitored by quantitative Oil-Red-O staining at day 16 (Figure 4). When peptides were incubated with cells during the early stage of differentiation, GPL and IY reduced significantly ($p<0.05$) the final lipid accumulation by 13.5±3.6% and 8.3±0.3%, respectively, while VIY had only a minor effect on the final lipid content (no significant $p >0.05$). However, none of the peptides tested affected significantly ($p >0.05$) the final lipid content when incubated during the second period of differentiation process.

3.3. Effect of marine cryptides on GPDH activity

The activity of GPDH, involved in TG synthesis, was measured on day 8 after the addition of 100 µM of GPL, IY, VIY, KY, VY and LKP during differentiation. We measured a really low GPDH activity in undifferentiated pre-adipocytes (data not shown). As shown in Figure 5, GPDH activity decreased by 33.7±10.6% ($p<0.05$) in HWP cells incubated with GPL compared with untreated cells. Cells treated with IY also had a GPDH activity significantly ($p<0.05$) decreased by 25.8±4.8%. On the other hand, there was no significant ($p>0.05$) change in the GPDH activity of HWP cells treated with other tested peptides (VY, VIY, KY or LKP).

3.4. Effect of marine cryptides on the expression of adipogenic marker genes

In order to assess appropriate genetic changes during the HWP cells differentiation, RT-PCR assay was first carried out for undifferentiated cells and cells after 8 days of differentiation, without peptide treatment. We checked that mRNA expression level of all genes tested increased as differentiation proceeded, supporting that the differentiation of pre-adipocyte was induced properly (data not shown).

We previously showed that GPL and IY decreased significantly the total lipid content and the GPDH activity indicating an action during early differentiation process. The effect of incubating HWP with GPL or IY was measured on the expression of markers of adipocyte differentiation (LPL, GLUT4, aP2 and AGT). In this study, we added the cryptide LKP as a negative control since no change on lipid content or GPDH activity was observed previously with this cryptide.

HWP were treated at day 8 with 100 µM of the above mentioned peptides. The effect of GPL, IY and LKP on mRNA expression of adipogenic genes during HWP differentiation is presented Figure 6. The mRNA expression of GLUT4 and aP2 were significantly down-regulated by approximately two-fold ($p<0.01$) after GPL treatment. When compared with untreated cells, the addition of GPL to the medium resulted also in a decrease of relative LPL and AGT mRNA expression to 0.81±0.09 ($p<0.05$) and 0.70±0.05 ($p<0.01$) respectively.

The mRNA expression level of LPL, GLUT4, aP2, and AGT were also down-regulated by IY treatment (Figure 6A, B, C, D) but in a lesser extent. However, LKP did not affect gene expression except for aP2 whose expression increased.

We then investigated whether, after 8 days of treatment during differentiation, GPL and IY could suppress adipogenesis through the PPARγ and C/EBPα pathways. GPL and IY

significantly down regulated mRNA expression of PPARγ (p<0.05) (Figure 6E) and C/EBPα (p<0.01) (Figure 6F). However, the effect of IY were weaker than the one of GPL: the relative mRNA expression of PPARγ and C/EBPα were decreased to 0.70±0.09 and 0.47±0.01, respectively, compared to the control cells after GPL treatment (Figure 6E, F), while relative mRNA expression of PPARγ and C/EBPα were decreased to 0.80±0.09 and to 0.78±0.06 when cells were incubated with IY.

4. Discussion

In our study, we investigated *in vitro* the impact of bioactive peptides derived from marine organisms on the proliferation and the differentiation of human white pre-adipocytes cells in order to identify their possible uses in the control of human body mass. All the cryptides chosen in our investigation were reported to have an antihypertensive potential by the inhibition of ACE activity. The link between inhibitors of ACE and weight is currently under investigation and targeting the adipocyte cell cycle seems to be one of the solutions proposed against obesity.

We first defined the cryptide concentration of study and decided to work with a quite high concentration (100µM) in order to discriminate quickly the most interesting peptides: those exerting an action on the development of adipocytes.

We demonstrated that all ACE inhibitory peptides could not affect the viability of HWP cells during proliferation and differentiation. Among 11 cryptides tested, 5 were found to be toxic for human adipocytes at a concentration of 100 µM: AKK, AP and VAP decreased preadipocytes viability while KW and VW exert their toxic effect during the differentiation stage. The decrease of cell numbers was probably due to the inhibition of cell division and/or induction of cell death by apoptosis in HWP. This was already demonstrated by a number of

natural products such as polyphenol compounds which were shown to inhibit preadipocytes proliferation and induce apoptosis. For example, quercetin and diphlorethohydroxycarmalol (DPHC) induced apoptosis in 3T3-L1 pre-adipocyte by decreasing mitochondria membrane potential, down-regulating poly (ADP-ribose) polymerase (PARP), Bcl-2 and activating caspase 3, Bax and Bak (Park et al., 2013; Rayalam et al., 2008). Kim et al. (2006) suggested that adipocytes deletion by apoptosis could be a contributor to body fat loss Thus, the use of AKK, AP and VAP might be an appropriate approach to address obesity through adipocyte deletion.

Our results also revealed that KW and VW exerted an antiproliferative effect on HWP during the differentiation period in contrast to the HWP in proliferation stage and more interestingly they decreased cell viability during early stage of differentiation. This action on the differentiation stage was already observed with an omega-3 fatty acid found in fish oil. Indeed, Kim et al. (2006) reported that docosahexaenoic acid (DHA) did not affect cell growth in 3T3-L1 pre-adipocytes. However, after initiation of differentiation, DHA treatment decreased cell viability with an increase in LDH release. They suggested that DHA treatment induced apoptosis in 3T3-L1 during mitotic clonal expansion, occurring during the first few days after initiation of differentiation. In fact, it was reported that two critical events occur during the early stage of differentiation: mitotic clonal expansion and an irreversible commitment to differentiation (Rayalam et al., 2008). We demonstrated that GPL, LKP, IY, KY and VIY have no effect on the viability of HWP neither during proliferation nor differentiation. So they did not target the adipocyte life cycle through controlling adipocyte hyperplasia. However, these peptides could act like botanical alkaloids on the commitment to differentiation. Indeed, berberine, which affects the viability of 3T3-L1 during proliferation and differentiation and the viability of HWP cells only during the differentiation, induced a

major inhibition of differentiation and finally suppressed adipocyte hypertrophy in both 3T3-L1 and HWP cells (Hu, Fahmy, Zjawiony, & Davies, 2010; Hu & Davies, 2009).

Therefore, the effect of GPL, IY, VIY, KY, VY and LKP on adipocyte differentiation was evaluated using Oil-Red-O staining, the measure of GPDH activity and the mRNA expression changes of adipocyte differentiation markers.

The Oil-Red-O staining demonstrated that GPL, IY and VIY significantly decreased final lipid content in HWP cells. This inhibition of lipid accumulation was associated with a decrease of GPDH activity, a marker of late adipocyte differentiation only for GPL and IY.

Since GPL and IY seem to affect at least partially adipocyte differentiation, we next investigated the expression of adipocyte specific genes at the same stage of cell cycle to determine their potential mechanism of action.

The adipocyte differentiation process is coordinated by two major classes of transcription factors, namely peroxisome proliferator-activated receptor γ (PPARγ) and CCAAT/enhancer binding proteins (C/EBPs) (Yim, Hosokawa, Mizushina, Yoshida, Saito, & Miyashita, 2011). These two key regulators of adipogenesis mediate the acquisition of adipocyte phenotype and act synergistically to promote adipogenesis (Rahman et al., 2008).

We measured in the current study the expression of LPL, GLUT4, aP2, AGT, PPARγ, and C/EBPα, genes in response to GPL and IY peptide treatment and observed a significant decrease of their expression levels during HWP cells differentiation between cryptide-treated groups and control-treated groups. Thus, the GPL and IY treatment of maturing HWP might inhibit at least partially adipogenic activity through the down regulation of PPARγ and C/EBPα.

Few previous studies reported some similar effect of peptides derived from other natural sources on adipocyte differentiation. For example, Kim et al. (2007) demonstrated that a tripeptide IQN, purified from a black soybean protein hydrolysate, inhibited adipogenesis of an adipose cell line derived from mouse embryo, through the inhibition of differentiation. More recently, Jung et al. (2011) showed that silk peptides were able to suppress the adipocyte differentiation in C3H10T1/2 and 3T3-L1 cell lines through inhibition of Notch pathway.

The sequences of our most active cryptides, GPL and IY, have been already identified as important in the regulation of various mechanisms. For example, the sequence GPL pertains to the glyproline family (Samonina, Ashmarin, & Lyapina, 2002), peptide family including proline containing linear peptides, whose most important effects are the suppression of some reactions of blood coagulation and platelet aggregation and the protection of gastric mucosa against various ulcerogenic factors. Moreover, the association of amino-acids from GPL and IY can be found in the sequence of insulin membrane receptor. GPLY forms a recognition signal for insulin receptor endocytosis (Berhanu, Anderson, Paynter & Wood, 1995). Thus, further work will be undertaken to measure the role of GPL and IY on the insulin pathway.

5. Conclusion

Despite exhibiting high inhibitory activity of ACE (Ben Henda et al., 2013), LKP and VY have no effect on the proliferation and the differentiation of adipocyte cells, indicating that the ability of marine cryptides to affect adipogenesis is not correlated with their ACE-inhibitory activity.

We showed in this work that some marine peptides can act on adipose mass by targeting various stages in the life cycle of human white adipocytes. AP, VAP and AKK may induce inhibition of growth of preadipocytes during proliferation stage while KW and VW could decrease the number of preadipocytes engaged into the differentiation process. Treatment with GPL or IY inhibited adipogenesis through down-regulating adipocyte specific transcription factors.

It is important now to check lower concentration of peptides and better characterize the molecular way of action of GPL and IY peptides. We also wish to investigate the GPLY tetrapeptide effect on human white adipocyte cycle, in order to evaluate potent similarities and make a link between insulin pathway and GPL and IY cryptides.

These encouraging findings demonstrate for the first time that some cryptides, easily obtained from marine food products, can target the human white adipocyte life cycle. Thus, we believe that these marine cryptides have potential to become associated preventing/treating obesity agents.

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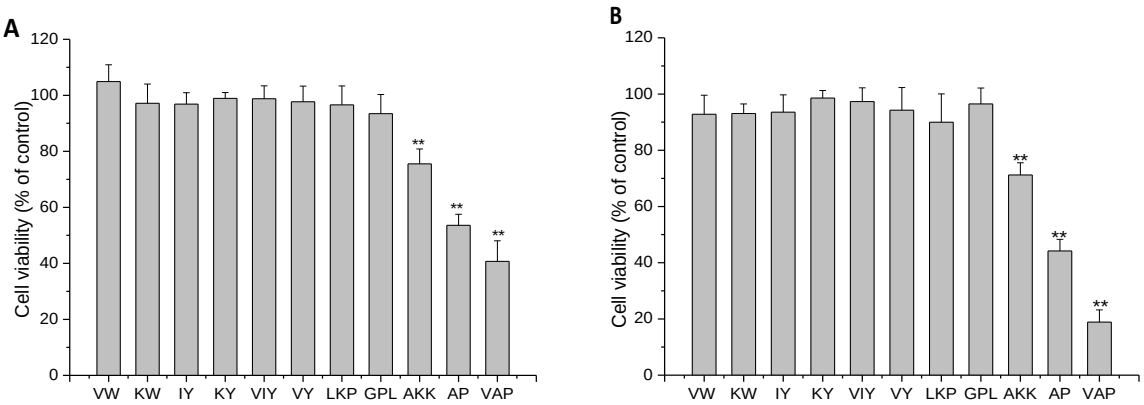
Table

Oligonucleotide	Sequences (5' to 3')
Human PPARγ-R	GGCGGTCTCCACTGAGAATA
Human PPARγ-F	GAGCCCAAGTTTGAGTTTGC
Human C/EBPα-R	TTTAGCAGAGACGCGCACATTCAC
Human C/EBPα-F	ATTGCCTAGGAACACGAAGCACGA
Human LPL-R	CTGGCATTGCAGGAAGTCTG
Human LPL-F	GCATCATCAGGAGAAAGACGA
Human aP2-R	TCTCTTTATGGTGGTTGATTTT
Human aP2-F	CAGTGTGAATGGGGATGTG
Human GLUT4-R	TTTCACCTCCTGCTCTAA
Human GLUT4-F	CGTCTTCCTTCTATTTGC
Human AGT-R	AGGCATAGTGAGGCTGGAT
Human AGT-F	CCTGGCTTTCAACACCTAC
Human β-actin-R	GCCTCGTCGCCCACATAG
Human β-actin-F	GCCGTCTTCCCCTCCATC

Table 1: Primer sequences used for Real time PCR

Figures



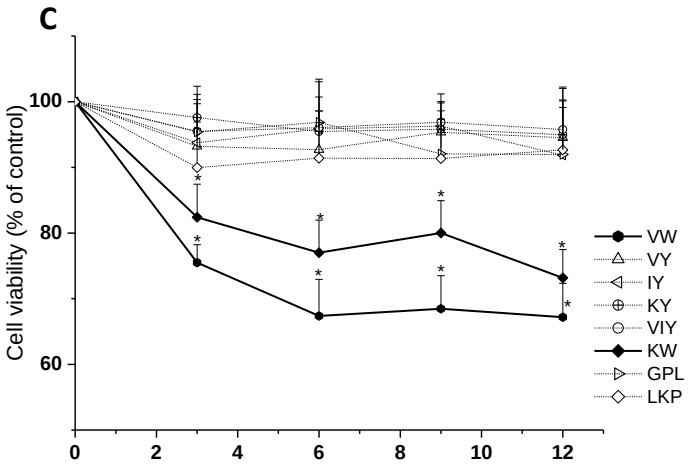


Figure 1

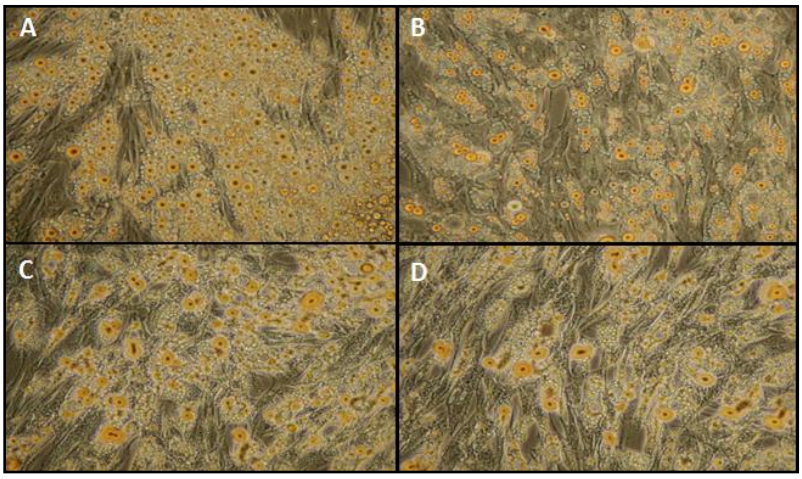


Figure 2

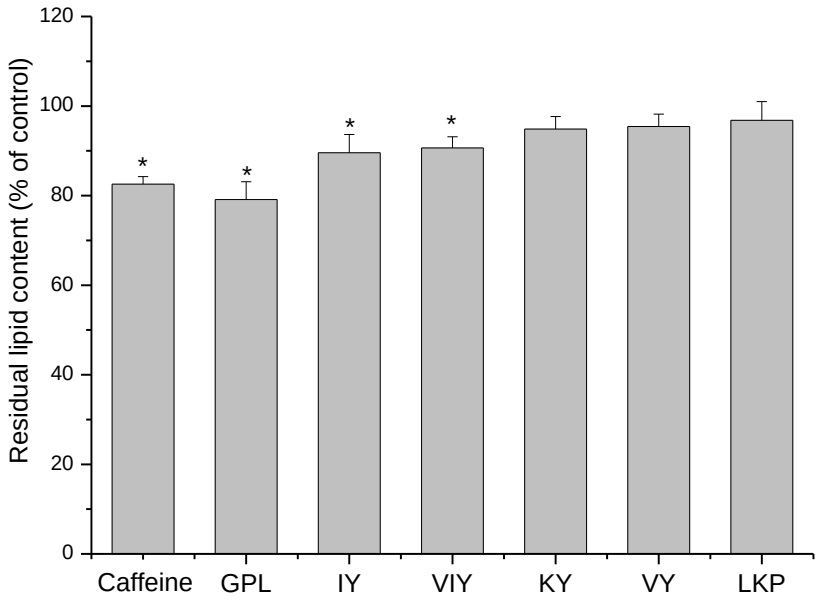

Residual lipid content (% of control)
120
100
80
60
40
20
0
*
*
*
*
Caffeine
GPL
IY
VIY
KY
VY
LKP

Figure 3

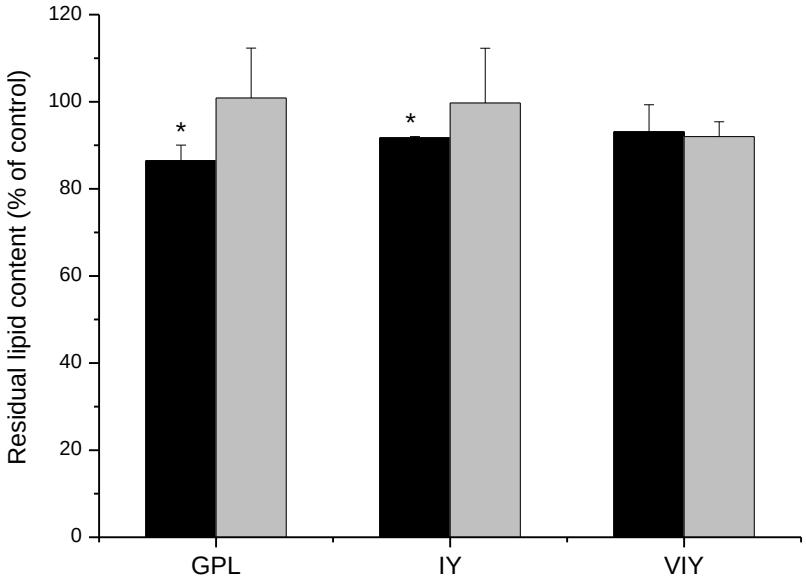


Figure 4

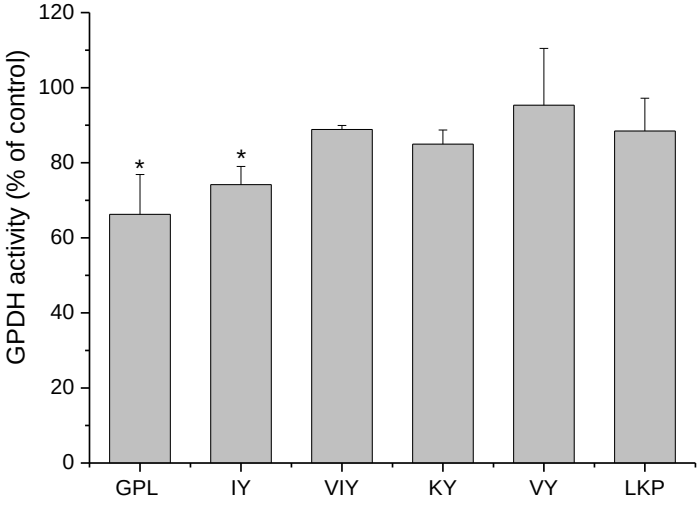
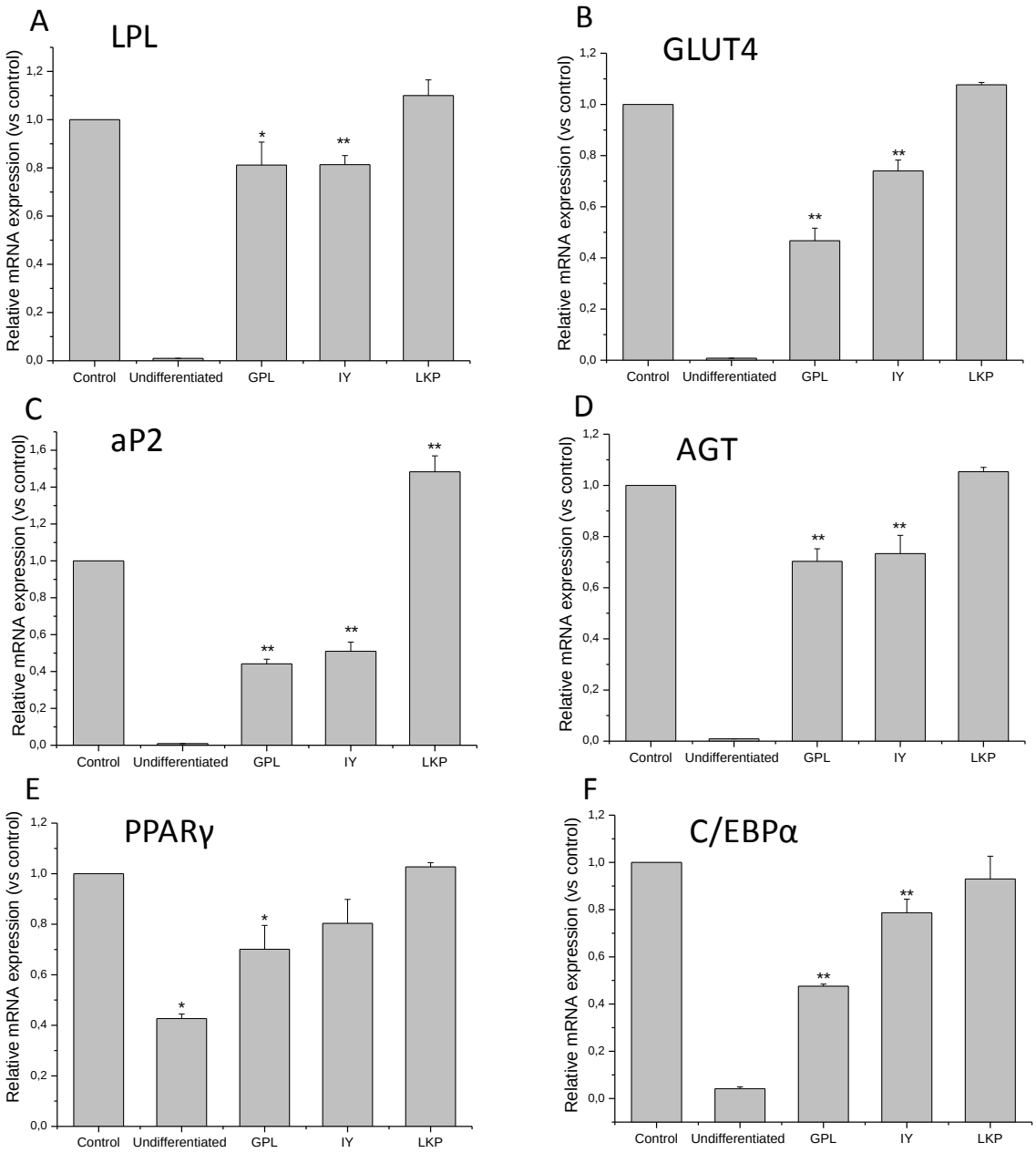


Figure 5



517

518 Figure 6

519

520 FIGURE CAPTIONS:

521 Figure 1:

522 Effect of cryptides on cell viability of HWP during proliferation after 24 h (A) and 72 h (B) of
523 treatment and after 3, 6, 9 and 12 days of differentiation (C). Cell viability was expressed as
524 percentage of non-treated cells (control). (A) and (B) Pre-confluent cells were treated with
525 100 µM of peptides (VW, KW, IY, KY, VIY, VY, VIY, LKP, GPL, AKK, AP, VAP) at 24 h
526 after cell plating. (C) Differentiation of HWP at confluence was induced and cells were
527 treated with 100 µM of peptides (VW, VY, IY, KY, VIY, KW, GPL, LKP) in appropriate
528 medium. Values are means ± SEM, *n*=24 replicates. *denotes significant difference compared
529 with non-treated group (p <0.05) and. ** (p<0.01). Cryopreserved cells were obtained from a
530 single donor.

531

532

533 Figure 2:

534 Effect of cryptides on lipid content of mature adipocytes. Cells were treated during
535 differentiation period (from day 0 to day 16) control (A), 100 µM of GPL (B), IY(C) and 100
536 µM caffeine (D).

537 Figure 3:

538 Effect of cryptides on residual lipid content of mature adipocytes. Values were presented as
539 percentage of non-treated cells (control) optical density. Cells were treated throughout the
540 differentiation period (from day 0 to day 16) with 100 µM of caffeine, GPL, IY, VIY, KY,
541 VY and LKP. Values are means ± SEM, *n*=12 replicates. *denotes significant difference

542 compared with non-treated group (p <0.05). Cryopreserved cells were obtained from a single
543 donor.

544

545 Figure 4:

546 Effect of cryptides on residual lipid content of mature adipocytes treated at different stage of
547 the differentiation process. Values were expressed as percentage of non-treated cells (control).
548 Cells were treated during differentiation process from day 0 to day 4 (■) or form day 4 to day
549 16 (■) with 100 µM of GPL, IY and VIY. Values are means ± SEM, n=12 replicates.
550 *denotes significant difference compared with non-treated group (p <0.05). Cryopreserved
551 cells were obtained from a single donor.

552

553 Figure 5:

554 Effect of cryptides on Glycerol-3-phosphate dehydrogenase (GPDH) specific activity of
555 HWP. GPDH activity was presented as percentage of the activity of non-treated cells
556 (control). Cells were treated during differentiation form day 0 to day 8 in the presence of 100
557 µM of GPL, IY, VIY, KY, VY and LKP. Values are means ± SEM, n=3 replicates. *denotes
558 significant difference compared with non-treated group (p <0.05). Cryopreserved cells were
559 obtained from a single donor.

560

561 Figure 6:

562 Effect of GPL, IY and LKP on mRNA expression of LPL (A), GLUT 4 (B), aP2 (C), AGT
563 (D), PPARγ (E) and C/EBPα (F) during HWP differentiation. Cells were treated during

differentiation from day 0 to day 8 in the presence of 100 μM of GPL, IY and LKP. The mRNA expression levels were measured by real time PCR and expressed as a ratio to non-treated cells (control) levels (=1.0) after normalization using the β-actin mRNA expression level. Values given are the means ±S.D. (n=3), ** and * denotes significant differences compared with non-treated cell ($p<0.01$) and ($p<0.05$). Cryopreserved cells were obtained from a single donor.