AMPK-derived peptides reduce blood glucose levels but lead to fat retention in the liver of obese mice

Nava Chapnik¹, Yoni Genzer¹, Avraham Ben-Shimon^{1,2}, Masha Y Niv^{1,2} and Oren Froy¹

¹Robert H. Smith Faculty of Agriculture, Food and Environment, Institute of Biochemistry, Food Science and Nutrition, The Hebrew University of Jerusalem, Rehovot 76100, Israel

²Fritz Haber Center for Molecular Dynamics, The Hebrew University, Jerusalem, Israel

Correspondence
should be addressed
to O Froy or M Y Niv

Emails

oren.froy@mail.huji.ac.il or
masha.niv@mail.huji.ac.il

Abstract

AMP-activated protein kinase (AMPK) is a regulator of energy balance at both the cellular and the whole-body levels. Direct activation of AMPK has been highlighted as a potential novel, and possibly safer, alternative to treat type II diabetes and obesity. In this study, we aimed to design and characterize novel peptides that mimic the α G region of the α 2 AMPK catalytic domain to modulate its activity by inhibiting interactions between AMPK domains or other interacting proteins. The derived peptides were tested *in vivo* and in tissue culture. The computationally predicted structure of the free peptide with the addition of the myristoyl (Myr) or acetyl (Ac) moiety closely resembled the protein structure that it was designed to mimic. Myr-peptide and Ac-peptide activated AMPK in muscle cells and led to reduced adipose tissue weight, body weight, blood glucose levels, insulin levels, and insulin resistance index, as expected from AMPK activation. In addition, triglyceride, cholesterol, leptin, and adiponectin levels were also lower, suggesting increased adipose tissue breakdown, a result of AMPK activation. On the other hand, liver weight and liver lipid content increased due to fat retention. We could not find an elevated pAMPK:AMPK ratio in the liver *in vivo* or in hepatocytes *ex vivo*, suggesting that the peptide does not lead to AMPK activation in hepatocytes. The finding that an AMPK-derived peptide leads to the activation of AMPK in muscle cells and in adipose tissue and leads to reduced glucose levels in obese mice, but to fat accumulation in the liver, demonstrates the differential effect of AMPK modulation in various tissues.

Key Words

- ▶ peptides
- ▶ metabolism
- ▶ AMPK
- ▶ obesity

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Introduction

Type II diabetes is among the main threats to human health in the twenty-first century, and in most countries, diabetes is now one of the leading causes of mortality through its effects on the cardiovascular system. Type II diabetes is characterized by insulin resistance and/or abnormal insulin secretion, resulting in elevated blood glucose levels (Zimmet *et al.* 2001). Current trends in drug prescribing for the treatment of insulin resistance indicate

a move away from agents that stimulate insulin secretion, such as sulfonylureas, toward agents that increase insulin sensitivity, such as the biguanide metformin and thiazolidinediones (Hardie 2007). Due to concerns regarding cardiovascular side effects of thiazolidinediones (Nissen & Wolski 2007), the US Food and Drug Administration (FDA) issued the toughest safety warning on two diabetes drugs, Avandia and Actos. Thus, nowadays metformin is

the first-line treatment for type II diabetes, in particular, in overweight and obese people and those with normal kidney function.

Recent findings indicate that metformin and thiazolidinediones indirectly activate the AMP-activated protein kinase (AMPK; Batandier *et al.* 2006). Direct activation of AMPK has been highlighted as a potential novel, and possibly safer, alternative for type II diabetes and obesity drug development (Hardie 2007). AMPK is a regulator of energy balance at both the cellular and the whole-body levels (Carling *et al.* 2011, Hardie 2011). Typically quiescent under normal conditions, AMPK is activated in response to hormonal signals or stress, such as hypoglycemia, strenuous exercise, and ischemia. The activation of AMPK is dependent on the ratio of AMP to ATP: while AMP activates AMPK, ATP has an inhibitory affect. When intracellular ATP levels are low, active AMPK inhibits numerous ATP-consuming pathways while activating the mobilization of intracellular energy stores to produce ATP. Conversely, when ATP levels are high, AMPK activity is inhibited, leading to the enhancement of energy storage pathways and increased energy usage for biosynthetic pathways (Kahn *et al.* 2005). Hypothalamic AMPK regulates energy intake by mediating the opposing effects of orexigenic and anorexigenic signals (Minokoshi *et al.* 2004). In peripheral tissues, AMPK is phosphorylated and activated under elevated AMP levels by liver kinase B1 (LKB1; Sanders *et al.* 2007) following hormonal signals, such as adiponectin (Yoon *et al.* 2006). Consequently, activated AMPK phosphorylates and, thus, inactivates acetyl-CoA carboxylase (ACC), the key enzyme in fatty acid synthesis. Reduced fatty acid synthesis results in mitochondrial fatty acid oxidation and activation of catabolic pathways (Yoon *et al.* 2006). *In vitro*, AMPK can be activated by 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR), an AMP analog, and inhibited by Compound C, an ATP-competitive inhibitor (Viollet *et al.* 2006). In the present study, we aimed to design novel AMPK modulators.

Most traditional drug discovery campaigns aim to develop small-molecular weight, orally available compounds, targeting catalytic or ligand-binding sites (Arora & Ansari 2009). Due to the constant decrease in productivity of drug design efforts (Booth & Zimmel 2004, Scannell *et al.* 2012), novel approaches, including targeting of protein–protein interactions (PPIs), are emerging in the drug discovery field. PPIs may be targeted by small molecules (Wells & McClendon 2007) or by peptidic and peptidomimetic compounds (Arora & Ansari 2009, Rubinstein & Niv 2009). We have recently designed

novel and highly specific kinase-inhibiting peptides (Niv *et al.* 2004, Wexler *et al.* 2005, Mack *et al.* 2008, Rubinstein & Niv 2009) for the prevention of insulin resistance and type II diabetes. In the present study, we designed peptides that mimic the α G region of the AMPK catalytic domain, a region typically involved in PPIs of kinases (Niv *et al.* 2004), to modulate its activity by inhibiting interactions with other AMPK domains or with interacting proteins, and examined the metabolic effects of such modulation.

Materials and methods

Animals, treatments, and tissues

Eight-week-old C57BL/6 or leptin-deficient *ob/ob* male mice were housed in a temperature- and humidity-controlled facility (23–24 °C and 60% humidity). Mice were entrained to a light–dark cycle of 12 h light:12 h darkness cycle for 2 weeks with food available *ad libitum*. After 2 weeks, mice were fed a high-fat (HF) diet for 10 weeks. The HF diet was based on soybean oil and palm stearin (fatty acid composition: C:12 0.3%, C:14 1.3%, C:16 55%, C:18 5.1%, C:18-1 29.5%, C:18-2 7.4%, and C:18-3 0.7%) and contained 22% w/w fat (42% kcal from fat). After 10 weeks of HF feeding, at an average body weight of ~31 g, C57BL/6 mice were injected subcutaneously with 0.4 mg/g body weight per day of myristoylated (Myr) or acetylated (Ac)-peptide or 0.4 mg/g body weight of Myr-peptide or Ac-peptide every other day for 12 days ($n=10$ in each group). The *ob/ob* mice were injected with 0.06 mg/g body weight of Myr-peptide for 12 days. Depending on the experiment, during the injection period, mice were given either a HF diet or regular chow. Daily food intake and body weight were monitored throughout the experiment. Mice were fasted for 10 h before tissue removal. Mice were anesthetized with isoflurane and blood, liver, gastrocnemius muscle, and white adipose tissue (WAT) were removed at the end of the experiment. After tissue removal, mice were humanely killed. Fasting blood glucose levels were determined using a glucometer (Optium Xceed; Abbott Laboratories). Tissues were immediately frozen in liquid nitrogen and stored at –80 °C until further analysis. The joint ethics committee (IACUC) of the Hebrew University and Hadassah Medical Center approved this study.

Tissue culture experiments

Mouse-derived C2C12 myoblasts were maintained in DMEM supplemented with 20% (v/v) bovine calf serum.

Differentiation of cells to myotubes was achieved by allowing the cells to reach confluence in 0.1% gelatin-coated flasks in DMEM supplemented with 10% (v/v) fetal calf serum (FCS). When the cells reached confluence, the medium was replaced with DMEM supplemented with 2% horse serum. Differentiation was achieved after 72–96 h. AML-12 hepatocytes were grown in DMEM supplemented with 10% (v/v) FCS and 200 mM glutamine for 3 days. Peptides were added to the medium at 100 μ M for 1 h, after which the cells were harvested and total and phosphorylated AMPK and ACC were measured by western blotting.

Serum separation and ELISA

Blood samples were kept at room temperature for 30 min for clotting and consequently centrifuged at 2000 *g* for 15 min. Serum was collected and stored at -80°C for further analysis. Serum hormone levels were determined for insulin (Mercodia, Uppsala, Sweden), corticosterone (Assaypro, St Charles, MO, USA), and leptin and adiponectin (R&D Systems, Inc., Minneapolis, MN, USA) using ELISA kits. Assays were performed according to the manufacturers' instructions.

Enzymatic colorimetric tests

Serum triglyceride, total cholesterol, HDL-cholesterol, alanine aminotransferase (ALT), and aspartate transaminase (AST) levels were determined using Cobas kits (Roche Diagnostics) and analyzed with a Roche/Hitachi analyzer (Roche Diagnostics). Assays were performed according to the manufacturers' instructions.

Homeostasis model assessment of insulin resistance

The insulin resistance index from fasting serum insulin and plasma glucose levels was determined using the homeostasis model assessment (HOMA) parameter: $\text{HOMA} = \text{fasting serum insulin } (\mu\text{U/ml}) \times \text{fasting plasma glucose (mg/dl)} / 405$.

Circular dichroism spectroscopy

Circular dichroism (CD) spectra of the peptide were recorded on a JASCO J-810 spectrophotometer (JASCO, Tokyo, Japan) using the supplied Spectra Manager software. The temperature was kept constant using a temperature-controlled water bath. Samples (400 μ l) containing 100 μ M peptide and 10 mM phosphate buffer were placed in 0.2 cm quartz cells (Starna, Atascadero, CA,

USA), and spectra were recorded in the wavelength range $\lambda = 195\text{--}260$ nm, with 5–10 accumulations for each measurement and a data pitch of 0.1 nm. Background CD spectra of the buffer were recorded and subtracted from each spectrum.

Peptide design

The peptide was derived from specificity-determining patches in AMPK catalytic domain, the α G helix in the human catalytic domain of AMPK $\alpha 2$ (accession P54646). The corresponding $^{215}\text{HVP}^{\text{TLFKKIRG}}^{225}$ sequence was amidated and conjugated either with Ac-Gly (Ac-peptide Ac-GHVP^{TLFKKIRG}-NH₂) or with Myr-Gly (Myr-peptide Myr-GHVP^{TLFKKIRG}-NH₂) as has been reported previously (Niv *et al.* 2004, Mack *et al.* 2008). The peptides were synthesized by GL Biochem at purity >95%.

Peptide structure prediction

Starting from an extended conformation, peptides were energy-minimized using conjugate gradient until the maximal force was below 1 kJ/mol or when no change in energy occurred. Subsequently, peptides were subjected to annealing molecular dynamics simulation of 300 ns with repeated cycles of the following heating and cooling sequence protocol: annealing time (ps): 0, 11, 70, 246, 257, 268, 327, 503, 522, 533, 592, 768, and 799; annealing temperature ($^{\circ}\text{C}$): 275, 290, 525, 290, 275, 290, 431, 290, 275, 290, 375, 290, and 275. The simulations were carried out with the GROMACS package version 4.5 (Pronk *et al.* 2013), in implicit solvent conditions (Onufriev *et al.* 2004), the AMBER96 force field, and virtual hydrogen representations that allow a large integration time step of 5 fs. No cutoffs were used; snapshots were saved every 1 ps. Bonds were constrained with the Lincs algorithm using Lincs order 2, with six iterations, to cancel out the possibility of the 'flying ice cube' phenomenon (Harvey *et al.* 1998). Snapshots along the trajectory at temperatures of 290–300 $^{\circ}\text{C}$ were clustered using the Gromos method, with backbone root mean square deviation (RMSD) cutoff of 1 $\text{\AA}$. The cluster-representative structures were sorted by the average energy of the best 15 poses of the cluster.

Lipid quantification

Lipid quantification in the liver was performed using the Folch reagent. Liver tissue samples weighing 100 mg were homogenized in a mixture of chloroform:methanol (2:1) and left shaking for 30 min. The samples were then

centrifuged at 2500 *g* for 30 min, and water was added. The samples were left open for evaporation and the test tubes were weighed. Lipid quantification in AML-12 cells was performed using Oil Red O staining. The cells were fixed in 10% formaldehyde in aqueous phosphate buffer overnight, washed with 60% isopropanol, and stained with Oil Red O solution (in 60% isopropanol) for 10 min. The cells were then repeatedly washed with water and destained in 100% isopropanol for 15 min. The optical density of the isopropanol solution was measured at 500 nm.

Western blot analysis

C2C12 or AML-12 cells were harvested, and liver and WAT samples (~200 mg) were homogenized in lysis buffer (20 mM Tris, 145 mM NaCl, 10% glycerol, 5 mM EDTA, 1% Triton-X, 0.5% Nonidet P-40, 100 μ M phenylmethylsulfonyl fluoride, 50 μ M NaF, and 1 mM sodium orthovanadate). The samples were run on a 10% SDS-polyacrylamide gel for AMPK, pAMPK (pThr172), ACC, and pACC (pSer79). After electrophoresis, proteins were semi-dry-transferred onto nitrocellulose membranes. Blots were incubated with anti-mouse AMPK/pAMPK and ACC/pACC, polyclonal antibody (Cell Signaling Technology, Beverly, MA, USA). Anti-mouse actin antibody (MP Biomedicals, Solon, OH, USA) was used to detect actin, the loading control. The membranes were washed and reacted with horseradish peroxidase (HRP)-conjugated anti-goat antibodies (Santa Cruz Biotechnologies). The immune reaction was detected by enhanced chemiluminescence (Santa Cruz Biotechnologies). Finally, bands were quantified by scanning and densitometry and expressed as arbitrary units.

Statistical analyses

All results are expressed as means $\pm$ S.E.M. Levels were assessed by one-way ANOVA, and a least-significant difference Tukey–Kramer *post hoc* analysis was used for comparison among the groups. For all analyses, the significance level was set at $P < 0.05$. Statistical analyses were carried out with the JMP 7.0.2 software (SAS Institute, Inc., Cary, NC, USA).

Results

Design of AMPK-derived peptides

With the goal of specifically modifying PPIs of AMPK, we designed a peptide mimicking the α G region of the

catalytic kinase domain (Fig. 1A and B). This region is typically involved in PPIs of kinases (Niv *et al.* 2004, Scholz *et al.* 2009). To evaluate the most stable conformations of the peptide in solution, we carried out molecular dynamics simulations of the Myr-peptide and Ac-peptide. We found that the most stable conformations of both the Myr-peptide and Ac-peptide were helical. The simulated structures perfectly fit the helical secondary structure of the native AMPK protein (Fig. 1C). The only deviation observed was the orientation of the first histidine residue, which is emphasized in stick representation in Fig. 1C. Excluding the histidine, the predicted lowest-energy structure of the largest conformational cluster was very similar to the native structure (the backbone and heavy atom RMSD of as low as 1 and 2.6 Å respectively). Independent simulations with different possible protonation states of the histidine (Ben-Shimon *et al.* 2013) yielded similar results (not shown). CD analysis of the peptide confirmed the helical conformation (Fig. 1D). We conclude that the derived peptide closely resembles the protein structure that it was designed to mimic, and the addition of the Myr or Ac moiety seems to influence only the orientation of the N-terminal (histidine) residue in the peptide.

Activity characterization of Myr-peptide and Ac-peptide in C2C12 myotubes

We next studied the effect of Myr-peptide and Ac-peptide on AMPK activity in a muscle cell line (C2C12 myotubes). Peptides were added to the medium at 100 μ M for 1 h, after which the cells were harvested and measured by western blotting for the levels of total AMPK and ACC and their phosphorylated forms. Both Myr-peptide and Ac-peptide elevated AMPK phosphorylation, which is reflective of its activation, with Myr-peptide having the highest activity. In parallel, the phosphorylation of the AMPK substrate, ACC, the rate-limiting enzyme in fatty acid synthesis, also increased ($P < 0.05$, Tukey's HSD) (Fig. 2). The effect of Myr-peptide was comparable to the activating effect of the *in vitro* reagent AICAR, namely a ~2.4-fold increase in AMPK phosphorylation (Fig. 2A) and a ~3.4-fold increase in ACC phosphorylation (Fig. 2B). Taken together, these results demonstrate that Myr-peptide and Ac-peptide activate AMPK in muscle cells.

Effect of Myr-peptide and Ac-peptide on body weight and food intake in mice

As AMPK activation leads to overall satiety and lipid oxidation, we next studied the effect of Myr-peptide and

Figure 1

Design and structure of the Myr-peptide. (A) The peptide was derived from the α G helix of the AMPK kinase catalytic domain α 2. AMPK (pdb 2H6D) is shown in gray and the region from which the peptide was derived in orange. (B) Peptide sequence of Myr-peptide. (C) Peptide structure prediction using simulated annealing molecular dynamics protocol (SAMDp). Three most energetically stable conformations obtained from molecular dynamics simulation of the peptide in implicit solvent are superimposed on the corresponding native AMPK region (orange) and

colored blue, pale blue, and cyan. RMSD of the peptide calculated with respect to the native AMPK region vs potential energy of the peptide as sampled during the SAMDp procedure is shown. The myristoyl group bonded to the first glycine residue, which is not part of the native AMPK sequence, was included in the simulations, but is not shown. (D) CD analysis of Myr-peptide. CD results obtained at 25, 37, and 50 °C confirmed the predicted helical content of this peptide.

Ac-peptide *in vivo* using diet-induced obese C57BL mice. Our results showed that obese mice treated with s.c. peptide injection of 0.4 mg/g body weight per day for 12 days gained significantly less weight ($P < 0.05$, Tukey's HSD) (Fig. 3A) with no reduction in average food intake (Fig. 3C). This reduction in body weight correlated with decreased adipose tissue weight (Fig. 3E). Injection of 0.4 mg/g body weight every other day led to an almost significant change in body weight ($P = 0.06$, Tukey's HSD) (Fig. 3B) with no change in food intake (Fig. 3D) and no reduction in adipose tissue weight (Fig. 3F). The activity of Myr-peptide was also confirmed in genetically obese leptin-deficient *ob/ob* mice. These mice treated with

s.c. peptide injection of 0.06 mg/g body weight per day (a typical dosage used for metformin) gained less weight than saline-injected controls (Fig. 3G) and consumed less food (Fig. 3H). Taken together, these results demonstrate that Myr-peptide and Ac-peptide lead to reduced adipose tissue weight and body weight, suggesting the activation of AMPK, at least in adipose tissue.

Effect of Myr-peptide on metabolic parameters

Our goal was to inject the lowest concentration possible and our preliminary results revealed that glucose levels change after every-other-day injections. In addition, as

Figure 2

Modulation of AMPK and ACC in C2C12 myotubes treated with AMPK-derived peptides or AICAR. (A) pAMPK:AMPK ratio. (B) pACC:ACC ratio. Peptides were added to the medium of C2C12 cells at 100 μ M for 1 h or 1 μ M AICAR, after which the cells were harvested and measured by western blotting for the levels of total AMPK and ACC and their phosphorylated forms. Different letters denote statistical significance ($P < 0.05$).

Myr-peptide and Ac-peptide were comparable in their effects, we carried out subsequent experiments only with Myr-peptide injected every other day. Injection of Myr-peptide every other day led to reduced levels of blood glucose (Fig. 4A), insulin (Fig. 4B), HOMA of insulin resistance (HOMA-IR) (Fig. 4C), HDL-cholesterol (Fig. 4D), triglycerides (Fig. 4E), and total cholesterol (Fig. 4F) ($P < 0.05$, Tukey's HSD). Corticosterone (Fig. 4G) levels did not differ between the saline-injected group and the

Myr-peptide-injected group ($P > 0.05$, Tukey's HSD). Leptin (Fig. 4H) and adiponectin (Fig. 4I) levels were lower in the Myr-peptide-injected group ($P < 0.05$, Tukey's HSD), correlating with the almost significant reduction in body weight and adipose tissue weight. By contrast, liver weight increased (Fig. 4J), although no change in liver functions, determined by serum AST:ALT ratio, was detected (Fig. 4K). However, liver lipid content increased significantly ($P < 0.05$, Tukey's HSD) (Fig. 4L). Serum parameters demonstrated a beneficial metabolic effect typical of AMPK activation. By contrast, liver fat accumulation suggested no AMPK activation in the liver.

To determine whether liver fat accumulation was a result of HF feeding during the peptide injection period, we fed mice a HF diet, and once peptide injections were commenced, mice were switched to a low-fat (LF) diet. At the end of the injection period (12 days), there was no difference in body weight between the saline-injected group and the Myr-peptide-injected group ($P > 0.05$, Tukey's HSD) (Fig. 5A). Serum glucose levels were not significantly different from day 0 in the saline-injected group. However, glucose levels were significantly higher in the Myr-peptide-injected group ($P < 0.05$, Tukey's HSD) (Fig. 5B) (see Discussion). Triglyceride levels were significantly lower in the Myr-peptide-injected group than in the saline-injected group ($P < 0.05$, Tukey's HSD) (Fig. 5C). As in the HF-fed mice, liver weight increased (Fig. 5A), although no change in liver functions, determined by serum AST:ALT ratio, was detected (Fig. 5D). In addition, liver lipid content increased significantly ($P < 0.05$, Tukey's HSD) (Fig. 5E). These results suggest that HF feeding during the injection period was not the reason for the accumulation of fat in the liver.

Activity of Myr-peptide in mouse liver and AML-12 hepatocytes

As liver fat accumulation does not typify AMPK activation, we next examined whether liver AMPK was activated as a result of Myr-peptide injections. Unlike in C2C12 cells (Fig. 2), liver pAMPK:AMPK ratio of the Myr-peptide-injected group was not different from that of the saline-injected group ($P > 0.05$, Tukey's HSD) (Fig. 6A). Moreover, pACC:ACC ratio of the Myr-peptide-injected group was even lower than that of the saline-injected group ($P < 0.05$, Tukey's HSD) (Fig. 6B), suggesting increased fat synthesis. To determine whether this holds for hepatocytes in general, we studied the effect of Myr-peptide on AMPK activation in a liver cell line (AML-12 hepatocytes). Myr-peptide was added to the medium at 100 μ M for 1 h, after

Figure 3

Effect of Myr-peptide or Ac-peptide injection on the body weight, food intake, and adipose tissue of mice fed a HF diet. (A) Mean body weight of mice injected every day for 12 days. The final body weight is shown in the inset. (B) Mean body weight of mice injected every other day for 12 days. The final body weight is shown in the inset. (C) Food intake of mice injected every day for 12 days. (D) Food intake of mice injected every other day for 12 days. (E) Mean adipose tissue weight of mice injected every day for 12 days. (F) Mean adipose tissue weight of mice injected every other day

for 12 days. (G) Mean body weight of *ob/ob* mice injected every other day for 12 days. (H) Food intake of *ob/ob* mice injected every other day for 12 days. After 10 weeks of HF feeding, at an average body weight of ~31 g, C57BL mice were injected subcutaneously with 0.4 mg/g body weight per day of Myr-peptide (Myr), Ac-peptide (Ac), or saline for 12 days or 0.4 mg/g body weight every other day for 12 days. The *ob/ob* mice were injected every other day with 0.06 mg/g body weight. During the injection period, mice were given a HF diet. $n = 10$ in each group. $*P < 0.05$.

which the cells were harvested and measured by western blotting for total AMPK and ACC and their phosphorylated forms. Myr-peptide did not elevate AMPK catalytic activity or ACC phosphorylation ($P > 0.05$, Tukey's HSD)

(Fig. 6C and D). These findings suggest that Myr-peptide does not activate AMPK in hepatocytes and together with the decreased ratio of pACC:ACC can explain liver fat accumulation.

Figure 4

Serum parameters, liver weight, and fat content in Myr-peptide-injected mice fed a HF diet. (A) Glucose. (B) Insulin. (C) HOMA-IR. (D) HDL-cholesterol. (E) Triglycerides. (F) Total cholesterol. (G) Corticosterone. (H) Leptin. (I) Adiponectin. (J) Liver weight. (K) AST:ALT ratio. (L) Liver lipid content. After 10 weeks of HF feeding, at an average body weight

of ~31 g, mice were injected subcutaneously with 0.4 mg/g body weight of Myr-peptide (Myr) or saline every other day for 12 days. During the injection period, mice were given a HF diet. All measurements were made on the last day of the experiment. $n = 10$ in each group. $*P < 0.05$.

Figure 5

Effect of Myr-peptide injected every other day for 12 days on the body, liver, and adipose tissue weight and serum parameters of mice fed a LF diet. (A) Mean body, liver, and adipose tissue weight. (B) Mean glucose levels on day 0 and day 12. (C) Triglycerides. (D) AST:ALT ratio. (E) Liver lipid content. After 10 weeks of HF feeding, at an average body weight of ~31 g, mice

were injected subcutaneously with 0.4 mg/g body weight of Myr-peptide (Myr) or saline every other day for 12 days. During the injection period, mice were given a LF diet. All measurements were made on the last day of the experiment. $n=10$ in each group. * $P<0.05$ and ** $P<0.05$ between groups.

Lipid accumulation in AML-12 hepatocytes

To enable the estimation of liver lipid accumulation, AML-12 cells were stained with Oil Red O after application of Myr-peptide and compared for the effect of metformin, lipid emulsion (LE), AICAR, Compound C, and their combinations (Fig. 6E). As expected, LE, but not metformin, caused lipid accumulation in AML-12 cells ($P<0.05$, Tukey's HSD). Myr-peptide caused fat accumulation in AML-12 cells, but to a lower degree

than LE. Similarly, AICAR, a direct activator of AMPK, also caused fat accumulation in AML-12 cells ($P<0.05$, Tukey's HSD). Metformin reduced the level of fat accumulation when combined with LE. Compound C, an AMPK direct inhibitor, reduced the levels of fat accumulation when combined with Myr-peptide or AICAR ($P<0.05$, Tukey's HSD) (Fig. 6E). These results indicate that Myr-peptide causes fat accumulation in hepatocytes.

Figure 6

Modulation of AMPK and ACC in liver and AML-12 hepatocytes treated with Myr-peptide. (A) Liver pAMPK:AMPK ratio. (B) Liver pACC:ACC ratio. (C) AML-12 cell pAMPK:AMPK ratio. (D) AML-12 cell pACC:ACC ratio. (E) Lipid content in AML-12 hepatocytes treated with metformin, Myr-peptide (Myr), lipid emulsion (LE), AICAR, Compound C (CC), and their combinations. (F) Muscle pAMPK:AMPK ratio. (G) WAT pAMPK:AMPK ratio. After 10 weeks of HF feeding, at an average body weight of ~31 g, mice were injected subcutaneously with 0.4 mg/g body weight of Myr-peptide (Myr) or saline every other day for 12 days. During the injection period, mice were given a HF diet. Liver, muscle, and WAT were removed and

measured by western blotting for the levels of total AMPK or ACC and their phosphorylated forms. Peptides were added to the medium of AML-12 cells at 100 μ M for 1 h, after which the cells were harvested and measured by western blotting for the levels of total AMPK and ACC and their phosphorylated forms. AML-12 cells were treated with 1 mM metformin, 100 μ M Myr-peptide, 0.1% LE, 1 μ M AICAR, 40 mM Compound C, and their combinations for 6 h. Lipid quantification in AML-12 cells was performed using Oil Red O staining. Different letters denote statistical significance ($P<0.05$). * $P<0.05$.

Activity of Myr-peptide in mouse muscle and adipose tissue

In light of the results obtained in the liver cell line, we studied AMPK activation in muscle and adipose tissue. Although AMPK was activated in C2C12 myotubes treated with Myr-peptide (Fig. 2), we could not detect such activation in muscle tissue *in vivo* ($P > 0.05$, Tukey's HSD) (Fig. 6F). By contrast, in WAT, increased AMPK phosphorylation was achieved in mice injected with Myr-peptide ($P < 0.05$, Tukey's HSD) (Fig. 6G). These results correlated with the reduced adipose tissue weight (Fig. 3).

Discussion

In this study, we designed a peptide based on the α G region of the $\alpha 2$ isoform of the kinase domain of AMPK and examined its effect on protein activity both *in vivo* and in cell culture. The α G region is typically involved in PPIs of kinases and has been used previously to design modulatory peptides (Niv *et al.* 2004, Wexler *et al.* 2005). We have also previously shown that α G region, together with the preceding H-J loop, undergoes rigidification upon substrate binding (Cheng & Niv 2010). In AMPK, in particular, homo-oligomerization and activation have been shown to be mediated by the α G region (Scholz *et al.* 2009). To evaluate the most stable conformations of the AMPK-mimicking peptide in solution, we carried out molecular dynamics simulations of Myr-peptide and Ac-peptide. Computational predictions of the most energetically stable conformations of peptides have evolved immensely with the advance of computational power and algorithms, tackling problems of increasing size and complexity (Dror *et al.* 2012, Ben-Shimon *et al.* 2013, Rizzuti & Daggett 2013). In the present study, we used a structure prediction procedure based on simulated annealing molecular dynamics in implicit solvent conditions and large integration time steps that efficiently reproduced the NMR structures of peptides (Ben-Shimon and Niv, unpublished). Our computational results, supported by CD experiments, indicate that the derived peptide closely resembles the protein structure that it was designed to mimic.

The effect of the AMPK-mimicking peptides was next explored *ex vivo*. A muscle cell line treated with the peptide exhibited the activation of AMPK alongside phosphorylation of its substrate, ACC. This activation was similar to that of AICAR, a known activator of AMPK. Obese mice injected every day with either Myr-peptide or Ac-peptide exhibited reduced body weight at 0.4 mg/g body weight.

Reduction of weight in mice injected every other day was achieved as well, albeit with a higher P value ($P = 0.06$), suggesting that the effective concentration to yield significant reduction in body weight is ~ 0.3 mg/g body weight per day. Interestingly, leptin-deficient obese mice exhibited significant reduction in body weight with fivefold less concentrated peptide. This result suggests that leptin may antagonize the effect of the peptide so that higher concentrations of peptide are needed to achieve reduction in body weight. Reduction in adipose tissue mass was observed in obese mice injected with the peptide while being fed a HF diet. Moreover, obese mice that were fed a LF diet while being treated with the peptide exhibited a greater reduction in fat depots. In light of the lack of change in food intake, these results suggest that the peptide indeed leads to the breakdown of fat depots. Indeed, AMPK is activated in adipose tissue, leading, in turn, to increased fatty acid oxidation and reduced fatty acid synthesis. However, when fat is supplied in the diet, it counteracts this breakdown.

Injection of Myr-peptide every other day led to reduced levels of blood glucose, insulin, and HOMA-IR, suggesting increased insulin sensitivity and reduced hepatic glucose output. Interestingly, when fed a HF diet during the injection period, blood glucose levels increased in both peptide-injected and saline-injected mice. However, the increase in blood glucose levels was lower in the peptide-injected group. When fed a LF diet during the injection period, blood glucose levels increased in the peptide-injected group, but not in the saline-injected mice compared with day 0. These results suggest that the peptide leads to a smaller increase in hepatic glucose output under HF feeding, and this hepatic glucose output is not influenced by the diet. It is plausible that the retention of lipids in the liver led to a constant hepatic glucose output regardless of the type of diet.

Importantly, our results suggest a dual effect *in vivo*. On the one hand, blood glucose levels, insulin levels, and insulin resistance index were reduced, as expected from AMPK activation. In addition, triglyceride, cholesterol, leptin, and adiponectin levels were lower, suggesting increased adipose tissue breakdown, also expected from AMPK activation. On the other hand, liver weight and liver fat content increased, indicating fat retention in the liver. The activation of AMPK in the liver leads to decreased gluconeogenesis and glucose output that could explain the reduction in blood glucose levels. However, we did not find an elevated pAMPK:AMPK ratio in the liver *in vivo* or in hepatocytes *ex vivo*. This finding suggests that the peptide does not lead to AMPK activation in

hepatocytes. Lack of AMPK activation would lead to lack of ACC phosphorylation and under HF feeding would lead to fat synthesis and accumulation in the liver. Indeed, the pACC:ACC ratio was reduced in the liver, suggesting increased fatty acid synthesis.

The effect of fat accumulation in the liver as a result of the peptide administration could also be observed in the hepatocyte cell line. Similarly, AICAR also led to fat accumulation in this cell line. Indeed, it has recently been shown that AICAR activates atypical protein kinase C together with AMPK in human hepatocytes, leading to an increase in lipogenic enzyme levels (Sajan *et al.* 2013). Nevertheless, other reports have shown significant reduction in hepatic triglyceride accumulation in both chow- and HF diet-fed animals treated with AICAR (Tomita *et al.* 2005, Henriksen *et al.* 2013).

Interestingly, muscle tissue exhibited no activation of AMPK after Myr-peptide treatment. These results contradict those obtained in C2C12 myotubes. The differences could stem from the different isoforms found in C2C12 myotubes vs gastrocnemius muscle. Indeed, it has been shown that $\alpha 2$ is the main isoform expressed in muscle tissue (Stapleton *et al.* 1996), whereas the isoform induced through C2C12 differentiation is $\alpha 1$ (Niesler *et al.* 2007). Similarly to muscle tissue, no AMPK activation was found in the liver. This finding could probably stem from the fact that both tissues express both $\alpha 1$ and $\alpha 2$ isoforms (Stapleton *et al.* 1996, Merrill *et al.* 2012). However, in adipose tissue, the predominant AMPK isoform expressed is $\alpha 1$ (Daval *et al.* 2005). The sequence corresponding to the HVPTLFKKIRG stretch, the $\alpha 2$ α G helix mimicked in the synthetic peptide in the present study, is HVPTLFKKICD in the $\alpha 1$ isoform. The difference in the last two amino acids of the peptide, cysteine aspartate vs arginine glycine, may lead to differential effects of the peptide on the two isoforms. The peptide may induce the activation of AMPK by interacting with a phosphatase that inactivates AMPK and, thus, disrupting inactivation. Alternatively, it may stem from the prevention of the domain–domain interactions within the AMPK, thus relieving self-inhibition and leading to increased activity. Alternatively, the effect may be due to the disruption of binding at the α G-helix that would promote the formation of homodimers or monomers instead of less-active higher oligomers (Scholz *et al.* 2009). Such interactions and disruptions thereof may very well be isoform specific. Discerning the mechanism of action of these novel peptides and their effects on $\alpha 1$ and $\alpha 2$ isoforms is the subject of a subsequent study. The difference in the metabolic response to agents that

activate AMPK in other cell lines and tissues is of extreme importance. These findings reiterate the differential effect that AMPK activation has on different tissues and the tissue-dependent and nutrition-dependent effects that may be elicited by AMPK modulators.

Conclusion

An AMPK-derived peptide leads to the activation of AMPK in adipose tissue and myotubes and to reduced adipose tissue mass and reduced glucose levels in obese mice. On the other hand, this peptide leads to fat accumulation in the liver. These results demonstrate the differential effect of AMPK modulation in various tissues.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

N C, Y G, and A B-S conducted the experiments and analyzed the data. O F, N C, M Y N planned the experiments and wrote the paper.

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