Received: 12 October 2018 | Revised: 27 March 2019 | Accepted: 2 April 2019

DOI: 10.1111/bph.14690

RESEARCH PAPER

The cholecystokinin receptor agonist, CCK-8, induces adiponectin production in rat white adipose tissue

Adrián Plaza | Beatriz Merino | Nuria Del Olmo | Mariano Ruiz-Gayo

Departamento de Ciencias Farmacéuticas y de la Salud, Facultad de Farmacia, Universidad CEU San Pablo, Madrid, Spain

Correspondence
Mariano Ruiz-Gayo, Departamento de Ciencias Farmacéuticas y de la Salud, Facultad de Farmacia, Universidad CEU San Pablo, Campus de Montepríncipe, Alcorcón, 28925 Madrid, Spain.
Email: ruigayo@ceu.es

Funding information
Ministerio de Ciencia, Innovación y Universidades, Grant/Award Numbers: BES-2013-063773, BFU2012-35353 and BFU2016-78556-R; Fundación Universitaria San Pablo CEU; European Regional Development Fund

Background and Purpose: A cholecystokinin (CCK) system has been identified in white adipose tissue (WAT). Nevertheless, the endocrine actions of CCK on WAT remain unknown. Our goal was to investigate the role of CCK in regulating the production of adiponectin, an adipokine expressed in WAT, which is pivotal in preserving energy homeostasis.

Experimental Approach: The effect of the bioactive CCK fragment CCK-8 on adiponectin production was studied both in vivo and in vitro. CCK-8 effects were characterized in rats treated with selective CCK_1 and CCK_2 receptor antagonists as well as in pre-adipocytes carrying the selective silencing of either CCK_1 or CCK_2 receptors. The influence of insulin on CCK-8 responses was also analysed.

Key Results: In WAT, CCK-8 increased plasma adiponectin levels and the expression of the adiponectin gene (*Adipoq*). In pre-adipocytes, CCK-8 up-regulated adiponectin production. CCK-8 effects were abolished by L-365,260, a selective CCK_2 receptor antagonist. CCK_2 receptor knockdown also abolished the effects of CCK-8 in pre-adipocytes. Moreover, in vitro CCK-8 effects were blocked by triciribine, a specific inhibitor of protein kinase B (Akt) and by the PPARγ antagonist T0070907. Silencing the expression of the insulin receptor inhibited CCK-8-induced *Adipoq* expression in pre-adipocytes. Furthermore, insulin potentiated the effect of CCK-8.

Conclusion and Implications: CCK-8 stimulates adiponectin production in WAT by acting on CCK_2 receptors, through a mechanism involving both Akt and PPARγ. Moreover, CCK-8 actions are only observed in the presence of insulin. Our results could have translational value in the design of new insulin-sensitizing therapies.

1 | INTRODUCTION

Cholecystokinin (CCK) is a postprandial gut hormone that stimulates the activity of the exocrine pancreas (Singer, 1987), inhibits gastric emptying (Debas, Farooq, & Grossman, 1975), and promotes short-term satiety by acting on CCK_1 receptors located both in abdominal vagal afferents and in brainstem areas (Beglinger & Degen, 2004). The CCK_2 receptors (also previously called gastrin receptors) are widely expressed in the CNS (Dufresne, Seva, & Fourmy, 2006) and, in the periphery; these receptors play a major role in maintaining the normal function of gastric mucosa (Chen et al., 2002). A recent study carried out in our laboratory has shown that activation of CCK_2 receptors facilitates the uptake of dietary triglycerides by white adipocytes, suggesting that CCK is involved in regulating homeostasis in white adipose tissue (WAT) (Plaza, Merino, Cano, et al., 2018; Plaza, Merino, Sánchez-Pernaute, et al., 2018). Related with that, studies carried out by Flatt's group have provided evidence of the effect of CCK in alleviating symptoms of insulin resistance (Irwin et al., 2012; Irwin,

Abbreviations: CCK, cholecystokinin; Sc-WAT, subcutaneous white adipose tissue; Vis-WAT, visceral white adipose tissues; WAT, white adipose tissue

Montgomery, O'harte, Frizelle, & Flatt, 2013), which supports the concept of an insulin/CCK drive of energy metabolism.

Adiponectin, which is the most abundant adipokine, plays a relevant role in glucose and lipid metabolism (Havel et al., 1996). In particular, plasma concentration of this hormone positively correlates with insulin sensitivity (Stern, Rutkowski, & Scherer, 2016), especially in obese individuals (Kadowaki et al., 2006; Kantartzis et al., 2005). Moreover, low adiponectin levels are often found in individuals with insulin resistance as well as in patients with Type 2 diabetes mellitus (Yatagai et al., 2003) and are now considered as an independent risk factor for diabetes and insulin resistance as well as for cardiovascular disease (Kumada et al., 2003). Also, the adiponectin/**leptin** ratio has been proposed as a valuable index to estimate WAT dysfunction and obesity-associated cardiometabolic risk (Frühbeck, Catalán, Rodríguez, & Gómez-Ambrosi, 2018). Mechanisms regulating adiponectin production continue to be a matter of debate (Fang & Judd, 2018). Thus, some studies have shown that **insulin** induces adiponectin gene expression and secretion (Pereira & Draznin, 2005), while others have reported an inhibition (Fasshauer, Klein, Neumann, Eszlinger, & Paschke, 2002). Glucocorticoids and **TNF-α** have been shown to inhibit adiponectin expression (Degawa-Yamauchi et al., 2005). In the case of leptin, this hormone has also been shown to increase adiponectin mRNA and protein (Singh et al., 2016). Nevertheless, the meaning of this finding is difficult to interpret as, unlike other adipokines, both adiponectin production and plasma concentration are often inversely associated with blood leptin levels (B. Lee & Shao, 2014). Hence, mechanisms regulating adiponectin production are a topic of research of increasing interest.

Taking into account (a) the role of CCK in regulating WAT homeostasis (Plaza, Merino, Cano, et al., 2018), (b) the existence of a complete CCK system in WAT (Plaza, Merino, Sánchez-Pernaute, et al., 2018), and (c) the already reported interaction between CCK and insulin in driving energy metabolism (Irwin et al., 2012; Irwin et al., 2013; Pathak, Flatt, & Irwin, 2018), we aimed at investigating the effect of the C-terminal bioactive fragment of CCK, **CCK-8**, on both adiponectin gene (*Adipoq*) expression and adiponectin production in rat WAT.

What is already known

- Both CCK_1 and CCK_2 receptors are expressed in white adipocytes.
- CCK receptors share signalling pathways with insulin receptors.

What this study adds

- Stimulation of CCK_2 receptors induces adiponectin gene (*Adipoq*) expression in adipocytes and promotes adiponectin production.
- The activation of CCK_2 receptors is ineffective in the absence of insulin.

What is the clinical significance

- CCK_2 receptor agonists could have translational value in the design of new insulin-sensitizing therapies.

2 | METHODS

2.1 | In vivo studies

2.1.1 | Animals

All animal care and experimental procedures conformed to the Guide for the Care and Use of Laboratory Animals (European Communities Council Directive 86/609/EEC) and were approved by the Ethics Committee of the Universidad CEU—San Pablo (refs Py103-15 and PROEX035/16). Animal studies are reported in compliance with the ARRIVE guidelines (Kilkenny, Browne, Cuthill, Emerson, & Altman, 2010) and with the recommendations made by the *British Journal of Pharmacology*. The in vivo studies were carried out in adult male Sprague–Dawley rats during the nocturnal period of the circadian cycle, under similar experimental conditions to those used in our laboratory to investigate the role of CCK-8 on other aspects of energy metabolism (Merino, Cano, Guzman, Somoza, & Ruiz-Gayo, 2008; Merino, Somoza, Ruiz-Gayo, & Cano, 2008; Plaza, Merino, Cano, et al., 2018). The dose of CCK-8 (10 $\mu g \cdot kg^{-1}$) was chosen on the basis of previous studies (Merino, Cano, et al., 2008; Plaza, Merino, Cano, et al., 2018). This dose did not significantly increase total CCK immunoreactivity in plasma and was able to activate **CCK receptors** in adipocytes (Plaza, Merino, Cano, et al., 2018). The time of CCK-8 administration (10 p.m.) was selected because CCK-8 has been shown to be more effective when administered earlier in the night (Kraly, 1981). Both plasma and WAT samples used in the current research were obtained from rats used in a recently published study (Plaza, Merino, Cano, et al., 2018) in which group sizes (n = 5–6) were calculated on the basis of an expected 20% reduction of food intake at the dose of 10-$\mu g \cdot kg^{-1}$ CCK-8, a statistical power >85%, and $P < .05$. The details of both the acute and chronic treatments with CCK-8 have been provided in an earlier publication (Plaza, Merino, Cano, et al., 2018).

2.1.2 | Pharmacological treatment

Treatment protocols are illustrated in Figure 1. Briefly, for acute treatment with CCK-8, 14-week-old male Sprague–Dawley rats (Harlan, Spain; 400–450 g) were individually housed under 12 hr light/12 hr dark (22°C; lights on at 08:00hr) with standard rodent chow (Teklad; 14% of energy from fat; 3.1 $kcal \cdot g^{-1}$; Harlan, Spain) and water ad libitum. The day of the experiment, rats were fasted from 09:00 until 18:00 and then fed ad libitum. In order to minimize the number of rats, the experiment was organized in sequential assays that were carried out at 1-week interval in the same animals, which were randomly assigned in each assay to either intervention or control groups. Operators were blinded to treatment assignment. Sulfated CCK-8 was selected as this CCK fragment has been shown to be the most

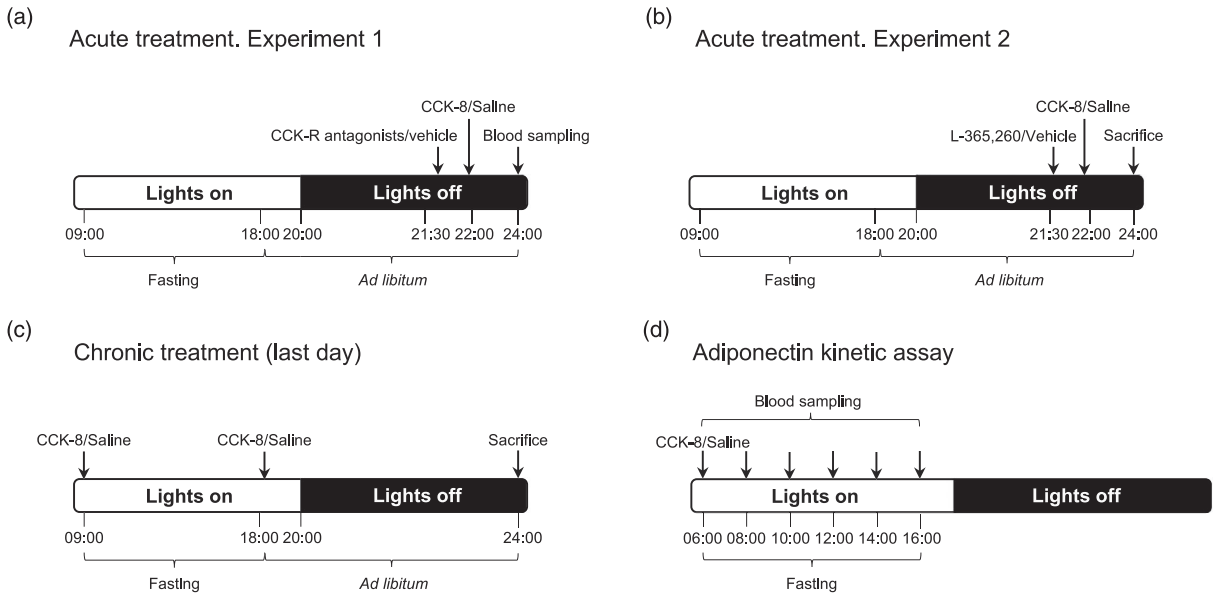

FIGURE 1 Experimental protocols used to analyse the effects of CCK-8 on adiponectin production (a–c), and the time course of the effect of CCK-8 on plasma adiponectin (d)

abundant form of CCK in rat and human plasma (Izzo, Brugge, & Praissman, 1984) that preserves the biological properties of full-size CCK (Miller & Gao, 2008). (1) The first assay aimed at identifying the CCK receptor subtype involved in the effect of 10-μg·kg^{-1} CCK-8 on plasma adiponectin levels. This dose of CCK-8 was chosen on the basis of previous studies aimed at identifying the role of CCK on the regulation of both feeding behaviour and energy balance (Cano, Merino, Ezquerra, Somoza, & Ruiz-Gayo, 2008; Merino, Cano, et al., 2008; Plaza, Merino, Cano, et al., 2018). Animals received at 21:30hr either saline (n = 12), **L-365,260** (a CCK_2 receptor antagonist; 1 mg·kg^{-1}; n = 12), or **SR-27,897** (a CCK_1 receptor antagonist; 0.3 mg·kg^{-1}; n = 12) subcutaneously. Thirty minutes later, rats were treated intraperitoneally with either saline (saline + saline, n = 6; L-365,260 + saline, n = 6; SR-27,897 + saline, n = 6) or 10-μg·kg^{-1} CCK-8 (saline + CCK-8, n = 6; L-365,260 + CCK-8, n = 6; SR-27,897 + CCK-8, n = 6). At 24:00hr, a sample of blood was obtained from the caudal vein after application of a mixture of lidocaine and prilocaine (EMLA; Figure 1a). (2) The second assay was designed to quantify adiponectin gene expression in adipose tissues and was performed in rats that were randomly assigned to receive either saline (n = 10) or L-365,260 (1 mg·kg^{-1}; n = 10) at 21:30hr. Five animals of each group received either saline or CCK-8 (10 μg·kg^{-1}) at 22:00hr. Animals were killed by decapitation, under inhalational anaesthesia with isoflurane, at 24:00hr, and visceral (Vis-WAT) and subcutaneous adipose tissues (Sc-WAT) were dissected and frozen in liquid nitrogen. Treatment with SR-27,897 was omitted in this case as this antagonist failed to modify plasma adiponectin levels in Experiment 1 (Figure 1b).

For chronic treatment, 12-week-old male Sprague–Dawley rats underwent chronic treatment (12 days; twice daily, at 09:00hr and 18:00hr) with either saline (n = 6) or CCK-8 (n = 6, 10 μg·kg^{-1}). On Day 12 of the treatment, animals were decapitated at 24:00hr, under inhalational anaesthesia with isoflurane (Figure 1c).

2.2 | Plasma adiponectin kinetics

Another group of 14-week-old male Sprague–Dawley rats was used to characterize the time course of CCK-8 on plasma adiponectin levels. Rats were treated with either saline (n = 6) or CCK-8 (n = 6, 10 μg·kg^{-1}) at 06:00 hr, food was removed, and blood samples were obtained by incision of the tail vein at 2-hr intervals until 16:00 hr (Figure 1d). Sampling was performed under local anaesthesia with EMLA.

2.3 | Plasma biochemistry

Glucose (GTM, Roche, Spain), triglycerides (Biolabo, France), and non-esterified free-fatty acids (NEFA; Wako Bioproducts, USA) were measured by spectrophotometric methods. Both plasma leptin and insulin were quantified by means of EIA. Data corresponding to these analyses have been already published by us (Plaza, Merino, Cano, et al., 2018) and are provided as Supporting Information. Plasma adiponectin (Abcam, UK) was analysed by EIA.

2.4 | Isolation of adipose stromal vascular cells and differentiation of pre-adipocytes

The procedure was carried out as previously described (Gil-Ortega et al., 2013; Plaza, Merino, Cano, et al., 2018). Briefly, Vis-WAT and Sc-WAT samples from 12-week-old untreated Sprague–Dawley rats, provided by the animal facility of the Universidad CEU—San Pablo, were chopped and then incubated for 1 hr in α-MEM containing 13.6-U·ml^{-1} type NB4 collagenase (Serva, Germany) and 20-μg·ml^{-1} type I DNAse (Roche, Spain). After enzymic digestion, samples were centrifuged at 300 x g (10 min, 25°C) and supernatants containing adipocytes discarded. The pellets containing the stromal vascular fraction were resuspended in 1-ml α-MEM, then filtered through a 37-μm

mesh, and centrifuged at 300 x *g* (10 min, 25°C). Pellets were treated with erythrocyte lysis buffer (155-mM NH_4Cl, 5.7-mM K_2HPO_4, 0.1-mM EDTA, pH = 7.3), then re-centrifuged under identical conditions, resuspended in α-MEM supplemented with 10% newborn calf serum (NCS, Sigma, Spain), 10-U·ml^{-1} penicillin–streptomycin (Life Technologies, Spain), and 0.25-μg·ml^{-1} amphotericin B (Life Technologies), plated in 12-well plates (40,000 cells·cm^{-2}), and cultured during 5–7 days. Differentiation of adipose-derived stem cells into pre-adipocytes was induced in differentiation medium (α-MEM, containing 2% NCS, 66-nM insulin, 1-nM triiodothyronine, 1-mM dexamethasone, 0.3-mM rosiglitazone, and 10-mg·ml^{-1} apotransferrin) for 7–9 days.

2.5 | Determination of adiponectin levels in pre-adipocytes treated with CCK-8

Visceral and subcutaneous pre-adipocytes were treated with CCK-8 (10^{-6} M) during 4 hr. After this time, the medium was replaced by CCK-free differentiation medium, and cells were collected immediately (CCK-8 group, *n* = 5), as well as after 1 hr (CCK-8 + 1 hr group, *n* = 5) or 2 hr (CCK-8 + 2 hr group, *n* = 5). A control group (*n* = 5) without CCK-8 treatment was used. Cells were homogenized in ice-cold buffer containing 0.42-M NaCl, 20-mM HEPES (pH 7.9), 1-mM $Na_4P_2O_7$, 1-mM EDTA, 1-mM EGTA, 1-mM DTT, 20% glycerol, 1-mg·ml^{-1} aprotinin, 1-mg·ml^{-1} leupeptin, 20-mM sodium fluoride, 1-mM trisodium orthovanadate, and 2-mM phenylmethylsulfonyl fluoride. The homogenates were frozen at −80°C, thawed at 37°C three times, and then centrifuged for 10 min at 4°C. Equal amounts of protein (50 μg) were mixed with Laemmli buffer (50-mM Tris pH = 6.8, 10% sodium dodecyl sulfate, 10% glycerol, 5% mercaptoethanol, and 2-mg·ml^{-1} blue bromophenol), then loaded on an SDS-PAGE gel, and submitted to electrophoresis. Proteins were transferred to nitrocellulose membranes (GE Healthcare, Spain) by using a Transblot apparatus (Bio-Rad, Spain). The membranes were blocked with 5% dried skimmed milk powder in Tween-PBS for 1 hr.

The antibody-based procedures used in this study comply with the recommendations made by the *British Journal of Pharmacology*. Primary mouse monoclonal antibody against full-length human adiponectin (ab22554; Abcam) was applied (at a dilution of 1:1,000) overnight at 4°C. After washing, an appropriate anti-mouse secondary antibody (sc-516102; Santa Cruz Biotechnologies, USA; dilution 1/5,000) was added for 1 hr at room temperature. Blots were washed, incubated in chemoluminescence reagents (ECL Prime; GE Healthcare), and bands detected using the ChemiDoc XRS+ Imaging System (Bio-Rad). In order to check the equal loading of samples, blots were re-incubated with β-actin monoclonal antibody (Affinity Bioreagents, USA). All immunoblotting procedures follow the editorial on immunoblotting and immunohistochemistry (Alexander et al., 2018).

2.6 | Analysis of *Adipoq* expression by RT-qPCR

Visceral and subcutaneous pre-adipocytes were incubated with CCK-8 (10^{-7}–10^{-5} M) for 2 hr, then washed with PBS, and preserved with Trizol (*n* = 5; samples were run in duplicate). Total RNA was extracted by using the Tri-Reagent protocol (Life Technologies). cDNA was then synthesized from 1-μg total mRNA by using a high-capacity cDNA RT kit (Bio-Rad). Quantitative RT-PCR was performed by using designed primer pairs (Integrated DNA Technologies, USA; see Table 1). SsoAdvanced Universal SYBR Green Supermix (Bio-Rad) was used for amplification according to the manufacturer's protocols in an ABI PRISM 7000 Sequence Detection System (Applied Biosystems, USA). All gene values were normalized to the housekeeping gene *18s* and *β-Actin*. The ΔΔC(T) method was used to determine relative expression levels of rat genes. Statistics were performed using the ΔΔC(T) method (Livak & Schmittgen, 2001).

2.7 | Silencing of CCK and insulin receptors in pre-adipocytes

Differentiated rat pre-adipocytes were transfected with siRNAs for *Cckar*, *Cckbr*, and *Insr* (s234619, s218025, and s128753, respectively; Life Technologies), as previously described (Plaza, Merino, Cano, et al., 2018). Briefly, for each well, siRNAs (40 nmol·L^{-1}) were diluted in 50-μl Opti-MEM (Life Technologies), incubated for 15 min (25°C), and then added to a solution containing 3-μl lipofectamine RNAiMAX (Life Technologies) diluted in 50-μl Opti-MEM. After 15 additional min (25°C), 100 μl of the mixture were added drop-wise to wells containing 400-μl Opti-MEM. The medium was replaced by differentiation

TABLE 1 Designed primer pairs used in this study

mRNA	Forward	Reverse
Rat *Adipoq*	5′-CCCTCCACCCAAGGAAACTT-3′	5′-CTTCCGCTCCTGTCATTCCA-3′
Rat *Pparg*	5′-CGTTCACAAGAGCTGACCCAATGG-3′	5′-ATAGTGGAAGCCTGATGCTTTATCCC-3′
Rat *Il6*	5′-AGAGACTTCCAGCCAGTTGC-3′	5′-ACTCCAGAAGACCAGAGCAGA-3′
Rat *Il1b*	5′-TCACTCATTGTGGCTGTGGA-3′	5′-TGTCGTTGCTTGTCTCTCCT-3′
Rat *Il10*	5′-CAGGACTTTAAGGGTTACTTGGGTTGC-3′	5′-AAACTCATTCATGGCCTTGTAGACACC-3′
Rat *Tnfa*	5′-TTCTCATTCCTGCTCGTGGC-3′	5′-CTCCGCTTGGTGGTTTGCTA-3′
Rat *18s*	5′-ACTCAACACGGGAAACCTCA-3′	5′-AATCGCTCCACCAACTAAGA-3′
Rat Actin	5′-CCCTCTGAACCCTAAGGCCAACCG-3′	5′-GTGGTGGTGAAGCTGTAGCCACGC-3′

medium after overnight incubation. Two days after transfection, the medium was changed to α-MEM containing 0.1% BSA; then CCK-8 (10^{-6} mol·L^{-1}) was added and incubated for 2 hr (37°C, 5% CO_2). The medium was removed, and mRNA was extracted as detailed above. Positive controls for transfection were performed by using Block-iT Alexa Fluor Red (Life Technologies; n = 6; samples were run in duplicate). A *Silencer Select* negative control (4390843, Thermo Fisher Scientific, Spain) was used.

2.8 | Inhibition of Akt with triciribine

Sc-WAT and Vis-WAT derived pre-adipocytes were pre-incubated with 10^{-5}-M **triciribine** in differentiation medium for 30 min and then incubated for 2 additional hr with 10^{-6}-M CCK-8 (n = 6; samples were run in duplicate). After this time, mRNA was extracted as previously described. The dose of triciribine was selected accordingly to previous studies describing **Akt** inhibition with triciribine in adipocytes (C.-L. Chang et al., 2010).

2.9 | Inhibition of PPARγ with T0070907

Sc-WAT and Vis-WAT derived pre-adipocytes were pre-incubated with 10^{-5}-M **T0070907** in differentiation medium for 30 min and then incubated for 2 additional hr with 10^{-6}-M CCK-8, and mRNA was extracted (n = 6; samples were run in duplicate). The dose of T0070907 was selected on the basis of published data describing **PPAR**γ antagonism by T0070907 in adipocytes (Bosco et al., 2017).

2.10 | Combined treatment of pre-adipocytes with CCK-8 and insulin

Sc-WAT and Vis-WAT derived pre-adipocytes were pre-incubated with differentiation medium without insulin for 3 hr and then incubated for 2 additional hr with insulin (10^{-9} or 10^{-7} M) and/or CCK-8 (10^{-7}–10^{-5} M; n = 6; samples were run in duplicate).

2.11 | Data and statistical analysis

The data and statistical analysis in this study comply with the recommendations on experimental design and analysis in pharmacology (Curtis et al., 2018). Individual effects within a given group were analysed by using either a t test or a one-way ANOVA, followed by Bonferroni post hoc test when F ANOVA achieved $P < .05$, and there was no significant variance in homogeneity. Individual group comparisons were made by using a two-way ANOVA followed by Bonferroni post hoc test when appropriate. Statistical significance was set at $P < .05$. Normal distribution and variance homogeneity were assessed by using the Brown–Forsythe test. Outliers were identified by using the ROUT method (Q = 1%; GraphPad Prism software). All statistics were performed using GraphPad Prism software (GraphPad Software Inc., USA).

2.12 | Materials

The CCK_1 receptor antagonist, SR-27,897 (1-[2-4-(2-chlorophenyl) thiazol-2-yl-aminocarbonyl]-indolylacetic acid), was kindly provided by Sanofi-Synthelabo, France (Poncelet et al., 1993). The CCK_2 receptor antagonist, L-365,260 [(3R-(+)-2,3-dihydro-1-methyl-2-oxo-5-phenyl-1*H*-1,4-benzodiazepin-3-yl)-*N*′-(3-methylphenyl)urea], was a gift of Merck Sharp and Dohme Research Laboratories, USA (R. Chang et al., 1989). Other chemicals were from Sigma (USA).

2.13 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to Pharmacology (Harding et al., 2017), and are permanently archived in the Concise Guide to Pharmacology 2017/18 (Alexander, Christopoulos et al., 2017; Alexander, Cidlowski et al., 2017; Alexander, Fabbro et al., 2017).

3 | RESULTS

3.1 | CCK-8 enhanced adiponectin gene expression in both visceral and subcutaneous adipose tissues as well as in pre-adipocytes

Figure 2a,c illustrates the effect of CCK-8 on *Adipoq* expression in subcutaneous and visceral WAT, respectively. *Adipoq* expression was induced by acute CCK-8 (10 μg·kg^{-1}) only in Sc-WAT, (Figure 2a), but was up-regulated by chronic treatment in both Sc-WAT and Vis-WAT. In terms of *Pparg*, CCK-8 induced its expression after chronic treatment in Sc-WAT (Figure 2b) and was without effect in Vis-WAT (Figure 2d).

The effect of CCK-8 on visceral and subcutaneous pre-adipocytes appears illustrated in Figure 2e–h. In these assays, CCK-8 induced *Adipoq* expression both in Sc-WAT (Figure 2e), and Vis-WAT (Figure 2g). *Pparg* expression was only induced by CCK-8 in Sc-WAT (Figure 2f), but not in visceral pre-adipocytes (Figure 2h).

The robust effect of CCK-8 on *Adipoq* expression led us to investigate the effect of CCK-8 on adiponectin production both in vivo and in vitro. Otherwise, the similar qualitative pattern of *Adipoq* and *Pparg* gene expression suggested the involvement of PPARγ on adiponectin regulation, and therefore, the effect of PPARγ antagonists on *Adipoq* expression was further investigated.

3.2 | CCK-8 increased plasma adiponectin levels as well as adiponectin production in pre-adipocytes

As shown in Figure 3a, plasma levels of adiponectin were increased by both acute and chronic treatments with CCK-8. Figure 3b illustrates the time course effect of CCK-8 on plasma adiponectin levels. Two-way ANOVA revealed an effect of CCK-8, which was found to be

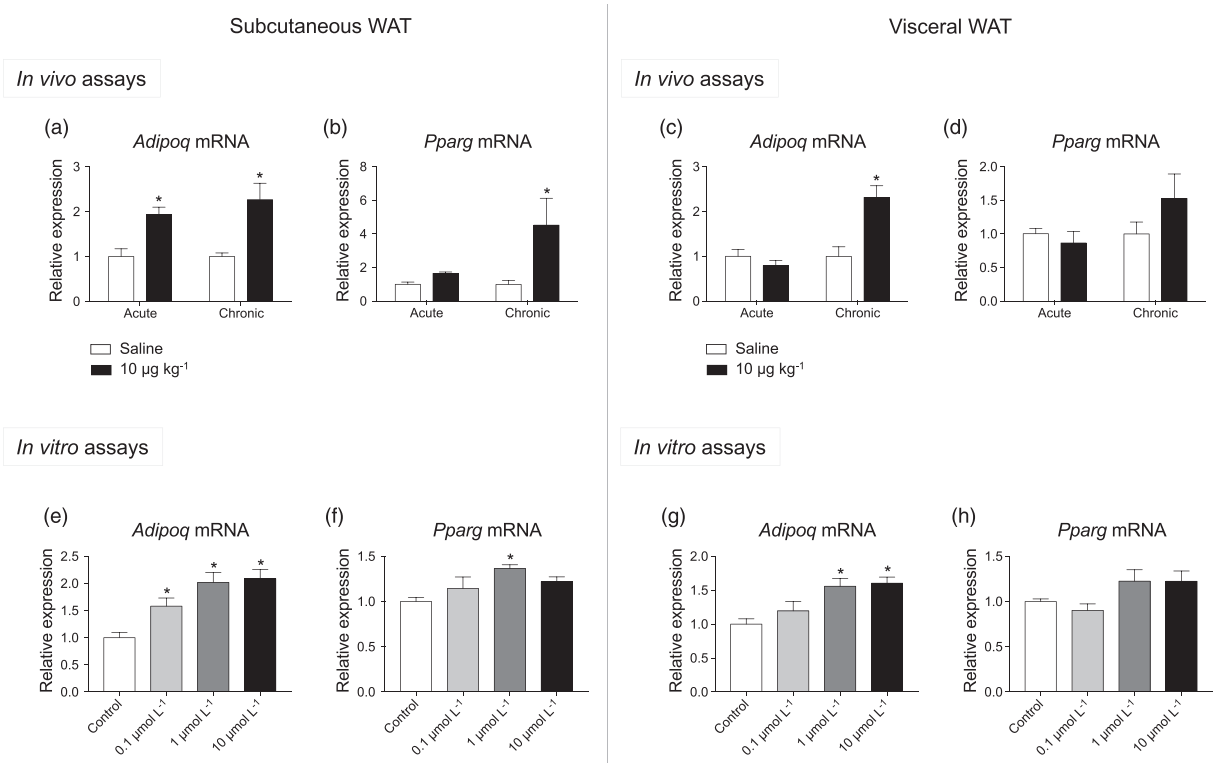

FIGURE 2 Effect of CCK-8 on adiponectin and PPARγ gene expression. Effect of acute and chronic administration of CCK-8 (10 µg·kg^{-1}) on adiponectin (*Adipoq*) and PPARγ gene (*Pparg*) expression in subcutaneous (a, b) and visceral WAT (c, e). The effect of CCK-8 was also assayed in pre-adipocytes. Panels (e) and (g) show the effect of CCK-8 on *Adipoq* expression in subcutaneous and visceral pre-adipocytes. Panels (f) and (h) illustrate the effect of CCK-8 on *Pparg* expression in subcutaneous and visceral pre-adipocytes. Values are means ± *SEM* (*n* = 5 for acute in vivo, *n* = 6 for chronic in vivo, and *n* = 6 for in vitro assays). *$P < .05$, significantly different from their respective controls; ANOVA with Bonferroni's test

maximal 4 hr after CCK-8 administration and reached basal values 2 hr later. However, adiponectin remained unaltered over the duration of the assay in saline-treated rats. Accordingly, the AUC of plasma adiponectin levels was significantly enhanced by CCK-8 (Figure 3c).

Figure 3d illustrates the time course of adiponectin content in subcutaneous pre-adipocytes incubated with 1-µM CCK-8. The effect of CCK-8 was maximal 1-hr after 4-hr incubation with CCK-8. In this case, adiponectin production remained unchanged in control cells (see Figure S1). Similar results were detected in visceral pre-adipocytes (Figure 3e), although in this case, CCK-8 effects were already significant immediately after 4-hr incubation with CCK-8. In both cases, the effect of CCK-8 was not observed in the CCK-8 + 2 hr group.

The results illustrated in Figure 3, taken together, show that CCK-8 triggers a transient increase of adiponectin production both in vivo and in vitro that seems to involve both Sc-WAT and Vis-WAT.

3.3 | The effect of CCK-8 on adiponectin production was mediated by CCK_2 receptors

In order to identify the CCK receptor subtype involved in regulating adiponectin production, in vivo (effect of CCK_1 and CCK_2 receptor antagonists) and in vitro assays (effect of *Cckar* and *Cckbr* silencing) were carried out. As illustrated in Figure 4a, one-way ANOVA revealed that the increase of plasma adiponectin triggered by CCK-8 (10 µg·kg^{-1}) was specifically abolished by the CCK_2 receptor antagonist, L-365,260, . Figure 4b shows the effect of the CCK_2 receptor antagonist L-365,260 on the up-regulation of *Adipoq* triggered by CCK-8. In this case, two-way ANOVA indicated a significant effect of CCK-8 and of L-365,260, as well as a significant interaction for CCK-8 × L-365,260.

Figure 4c,d illustrates the effects of silencing *Cckar*/*Cckbr* on *Adipoq* and *Pparg* expression in subcutaneous pre-adipocytes. In this assay, the effect of CCK-8 was specifically abolished in pre-adipocytes carrying the *Cckbr* deletion, for *Adipoq* and *Pparg* expression. In visceral pre-adipocytes, silencing *Cckbr* expression also abolished CCK-8 effects on *Adipoq* expression (Figure 4e).

3.4 | The inhibition of PKB by triciribine as well as the inhibition of PPARγ by T0070907 abolished the effect of CCK-8 on adiponectin gene expression

In order to investigate the involvement of the Akt signalling pathway on CCK-8-induced expression of *Adipoq* and *Pparg*, the effect of the Akt inhibitor triciribine was investigated. Triciribine abolished the

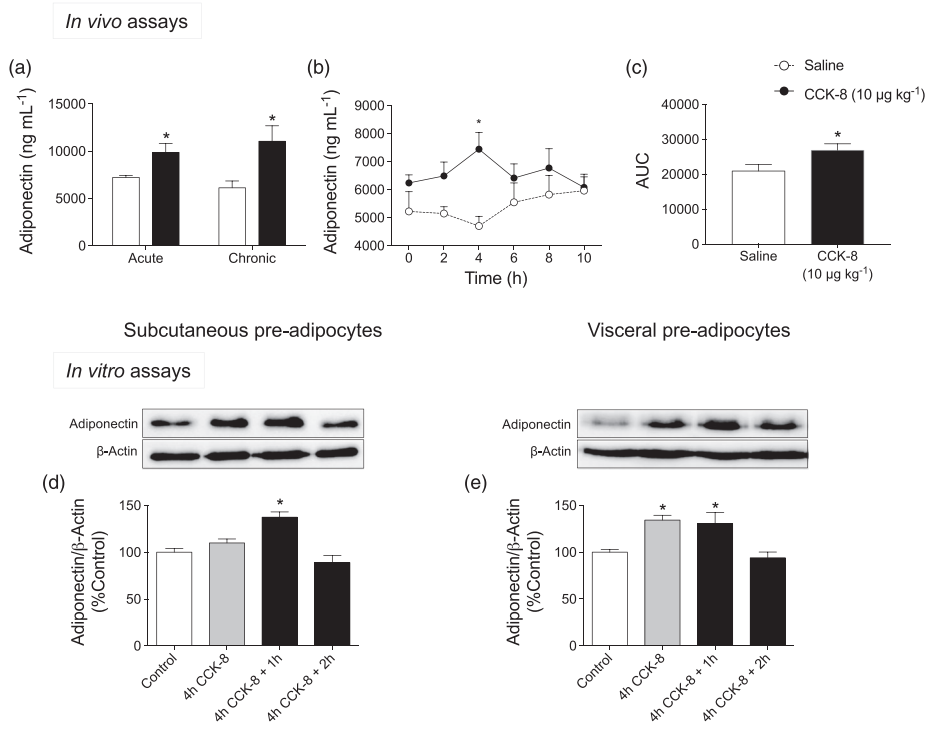

FIGURE 3 Effect of CCK-8 on adiponectin levels. (a) Effect of both acute and chronic administration of CCK-8 (10 μg·kg^{-1}) on plasma adiponectin concentration. (b) Time course of plasma adiponectin levels after acute treatment with CCK-8 (10 μg·kg^{-1}). This assay was carried out between 06:00 hr, when CCK-8 was administered, and 16:00 hr. (c) AUC corresponding to the time course of plasma adiponectin levels plotted in panel (b). Panels (d) and (e) illustrate the effect of 10-μM CCK-8 on adiponectin release in subcutaneous and visceral pre-adipocytes, respectively. Values are means ± *SEM* (n = 6 for in vivo and n = 5 for in vitro assays). *P < .05, significantly different from their respective controls; ANOVA with Bonferroni's test

effect of CCK-8 on *Adipoq* (Figure 5a) as well as on *Pparg* (Figure 5b) expression in subcutaneous pre-adipocytes. In visceral pre-adipocytes, triciribine also blocked the effect of CCK-8 on *Adipoq* expression (Figure 5c).

The involvement of PPARγ on *Adipoq* expression was assessed by using the selective PPARγ antagonist T0070907. As illustrated in Figure 5d,e, T0070907 antagonized the effect of CCK-8 on *Adipoq* expression in subcutaneous, as well as in visceral pre-adipocytes (Figure 5f; one outlier value was removed).

3.5 | The effect of CCK-8 on adiponectin gene expression requires the activation of the insulin receptor

The effect of CCK-8 was analysed in pre-adipocytes after silencing the expression of the **insulin receptor** gene (*Insr*) as well as in the presence of different concentrations of insulin. As illustrated in Figure 6a,b, the effect of CCK-8 on *Adipoq* expression was abolished by *Insr* silencing in both subcutaneous and visceral pre-adipocytes. Figure 6c and d shows the CCK-8 concentration-effect plots in the presence of different concentrations of insulin. In subcutaneous pre-adipocytes (Figure 6c), two-way ANOVA revealed a significant effect of insulin and of CCK-8, as well as a significant interaction for insulin × CCK-8. In visceral pre-adipocytes (Figure 6d), two-way ANOVA also revealed significant effects of insulin, and CCK-8, as well as a significant interaction for insulin × CCK-8.

4 | DISCUSSION

Our study showed that CCK-8, by acting on CCK_2 receptors, promoted the expression of *Adipoq* in subcutaneous and visceral WAT and, consequently, CCK-8 increased plasma adiponectin levels. Moreover, CCK-8 effects appeared to be insulin-dependent and, in the case of subcutaneous WAT, linked to the activation of PPARγ. Taken together, our findings support the concept, already suggested by previous studies carried out by our group and others (King, Yang, Huesman, Rider, & Lo, 2015; Plaza, Merino, Cano, et al., 2018; Plaza, Merino, Sánchez-Pernaute, et al., 2018), that both CCK and CCK binding sites have a relevant role in modulating WAT homeostasis, particularly in the case of Sc-WAT.

The main finding of our study deals with the increase of plasma adiponectin triggered by CCK-8. Such an effect was due to the activation of CCK-2 receptors in adipocytes, since (a) CCK-8 induced *Adipoq* expression in WAT as well as in pre-adipocytes, (b) CCK-8 increased adiponectin immunoreactivity in pre-adipocytes, and (c) CCK-8

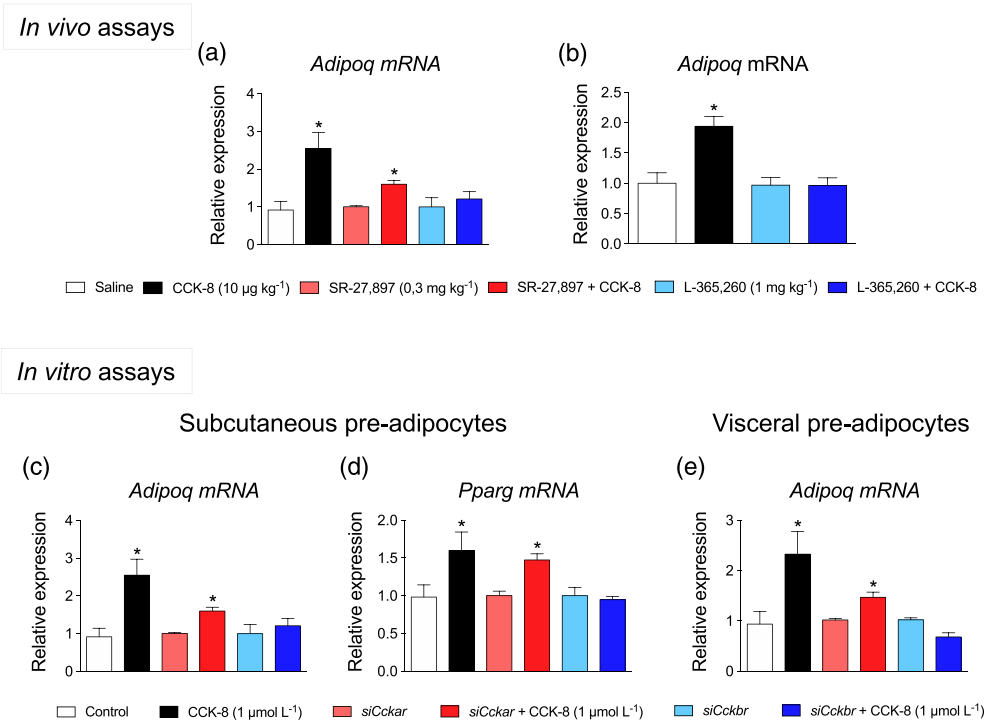

FIGURE 4 Effect of CCK receptor antagonists and silencing of CCK receptor genes on adiponectin production. (a) Effect of antagonists of CCK_1 receptors (SR-27,897) or CCK_2 receptors (L-365,260) on plasma adiponectin concentration. (b) Effect of L-365,260 on *Adipoq* expression in Sc-WAT from rats treated with CCK-8. (c, d) Effect of *Cckar* and *Cckbr* silencing on *Adipoq* and *Pparg* expression in subcutaneous pre-adipocytes. (e) Effect of *Cckar* and *Cckbr* silencing on *Adipoq* expression in visceral pre-adipocytes. Values are means ± *SEM* (*n* = 6). **P* < .05, significantly different from their respective controls; ANOVA with Bonferroni's test

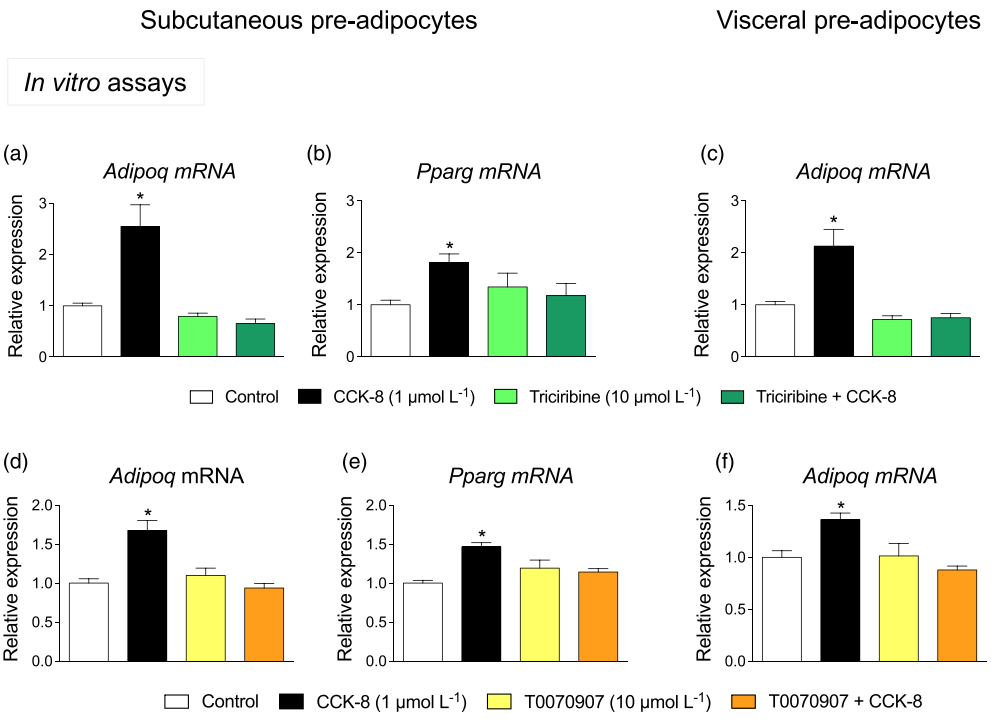

FIGURE 5 Effect of triciribine (an Akt inhibitor) and T0070907 (a PPARγ antagonist) on adiponectin gene (*Adipoq*) expression. Inhibition by triciribine of *Adipoq* (a) and *Pparg* expression (b) induced by CCK-8 in subcutaneous pre-adipocytes. *Adipoq* expression was also abolished by triciribine in visceral pre-adipocytes (c). T0070907 also blocked the effect of CCK-8 on *Adipoq* (d, f) and *Pparg* expression (e). Values are means ± *SEM* (*n* = 6). **P* < .05, significantly different from their respective controls; ANOVA with Bonferroni's test

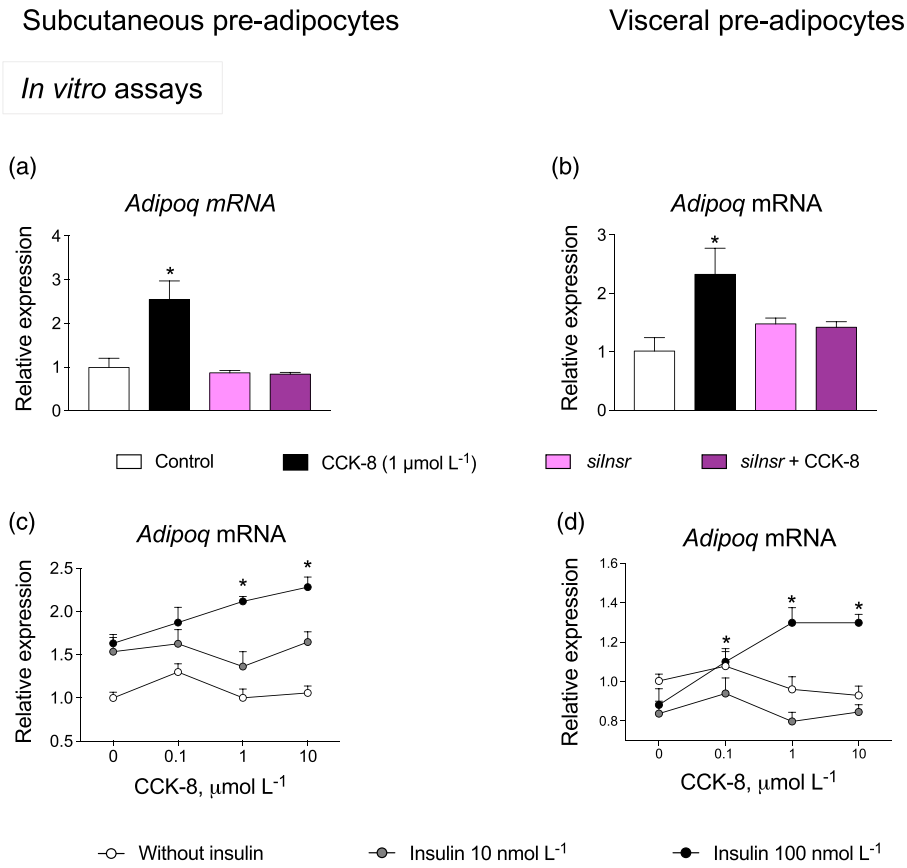

FIGURE 6 Insulin and insulin receptors are involved in the adiponectin gene (*Adipoq*) expression induced by CCK-8. Effects of silencing of insulin receptors on *Adipoq* expression in subcutaneous (a) and visceral pre-adipocytes (b). Concentration-effect plots of CCK-8 on *Adipoq* expression. Experiments were carried out in either the presence or absence of insulin in subcutaneous (c) and visceral pre-adipocytes (d). Values are means ± *SEM* ($n = 6$). *$P < .05$, significantly different from their respective controls; ANOVA with Bonferroni's test

effects were abolished by both genetic and pharmacological inactivation of CCK_2 receptors. Considering that CCK is physiologically released after meals, our data suggest that the effect of CCK in regulating adiponectin synthesis would be perceptible during postprandial periods and would contribute to maintenance of plasma adiponectin levels. This hypothesis is compatible with the circadian oscillation of plasma adiponectin in humans, characterized by a nocturnal decline reaching a nadir in the early morning, and a further rise coincident with the first meal of the day, which tends to stabilize until the following nocturnal decrease (Gavrila et al., 2003; Scheer et al., 2010). In our experiments, plasma adiponectin concentration reached the highest level at 4 hr after CCK-8 administration, which supports the concept of a long-lasting effect of CCK-8 in regulating adiponectinaemia. Nonetheless, it has to be noted that the kinetic assay illustrated in Figure 3b was carried out during the low activity period of the rat circadian cycle, a circumstance that limits the meaning of this result, and would explain the fact that plasma adiponectin reached lower values in this assay than when CCK-8 was administered at 10 p.m.

Another interesting finding deals with the preservation of the effect of CCK-8 on adiponectin production observed in chronically treated rats, which suggests that CCK-8 did not induce tolerance in adipocytes. In fact, chronic CCK-8 is more effective in promoting *Adipoq* expression in Vis-WAT than acute CCK-8. In any case, it has to be noted that the inhibition of food intake, together with the decrease of body weight triggered by CCK-8 (Plaza, Merino, Cano, et al., 2018), might contribute to the increase of adiponectin plasma levels (Reinehr, Roth, Menke, & Andler, 2004). At this point, it has to be highlighted that chronic CCK-8 led to a decrease of leptin plasma levels (Plaza, Merino, Cano, et al., 2018) that would rather limit the effect of CCK-8 on *Adipoq* expression, as leptin has been shown to induce the expression of adiponectin (Frühbeck et al., 2017; Singh et al., 2016). A relevant circumstance that deserves attention is the fact that the effect of CCK-8 on *Adipoq* expression seems to be slightly more intense in Sc-WAT than in Vis-WAT. This finding is of interest as subcutaneous pre-adipocytes produce and release more adiponectin than visceral pre-adipocytes (Baglioni et al., 2012), and, therefore, CCK_2 receptor agonists might be useful drugs to stimulate adiponectin production in physiopathological situations, such as obesity, characterized by a decrease of plasma adiponectin. Some studies have already identified a decrease of adiponectin expression in isolated subcutaneous pre-adipocytes from obese individuals (Degawa-Yamauchi et al., 2005). This topic has been reviewed by Lafontan and Girard (2008). Otherwise, the similar pattern of

expression of *Adipoq* and *Pparg* genes observed in the current study is compatible with the well-characterized link between *Adipoq* expression and PPARγ activity (Banga et al., 2009), and with the role of adiponectin on insulin-sensitizing therapies based on PPARγ agonists (Li et al., 2018; Tripathy et al., 2013). In this respect, our finding suggests that a pathway involving CCK receptors, PPARγ, and adiponectin could be interesting to investigate in developing of new insulin-sensitizing therapies. Interestingly, CCK-8 apparently promoted an anti-inflammatory response as it tended to repress the expression of the pro-inflammatory cytokines **IL-6**, **IL-1β**, and TNF-α in both Sc-WAT and Vis-WAT (see Figure S2). This effect would be consistent with the known anti-inflammatory effect of adiponectin (Frühbeck et al., 2017) and could contribute to an improvement in insulin sensitivity (Blüher, 2016).

The finding that triciribine blocks the effect of CCK-8 on adiponectin expression further confirms the importance of Akt in the CCK_2 receptor-mediated responses in WAT (Plaza, Merino, Sánchez-Pernaute, et al., 2018). The role of Akt in regulating adiponectin gene expression has not yet been fully elucidated, although Akt-mediated phosphorylation of **FoxO1** has been proposed to mediate the nuclear exclusion of this transcription factor. FoxO1 is a member of the forkhead box O transcription factor family that reduces the activity of the PPARγ-responsive element, which is necessary for the adiponectin gene transcription (Fan et al., 2009; Tzivion, Dobson, & Ramakrishnan, 2011). Thus, Akt activation might indirectly promote PPARγ-responsive element activity. The plausibility of this mechanism is stressed by the finding that CCK-8 also induced *Pparg* expression in subcutaneous WAT. Moreover, *Adipoq* expression was abolished by the PPARγ antagonist, T0070907 (G. Lee et al., 2002). All these findings are consistent with the pivotal role assigned to PPARγ as an upstream master regulator of *Adipoq* expression (Shao et al., 2016). As triciribine also antagonized the effect of CCK-8 on *Pparg* expression, one could hypothesize that both Akt and PPARγ would be integral to the mechanism that account for the regulation of CCK_2 receptor-mediated expression of adiponectin in subcutaneous adipocytes. Moreover, CCK_2 receptors can also activate the ERK1/2 pathway (Höcker, 2004), which regulates the PPARγ–adiponectin axis (Maeda et al., 2001; Yamauchi et al., 2001). Nevertheless, in the case of visceral adipocytes, molecular mechanisms governing *Pparg* expression seem to be independent of CCK_2 receptors.

In an effort to further identify the mechanism of action of CCK-8, the relationship between CCK_2 receptors and insulin receptors was explored. The finding that *Adipoq* expression was not induced by CCK-8 in pre-adipocytes carrying a deletion of the insulin receptor gene suggests a functional interaction between CCK_2 receptors and insulin receptors. This possibility was further strengthened by the synergism observed between CCK-8 and sub-threshold concentrations of insulin. From our data, we cannot identify the molecular mechanism that could eventually account for such an interaction. Although in vivo studies suggest that insulin acts as a negative regulator of adiponectin synthesis and/or secretion (Basu, Pajvani, Rizza, & Scherer, 2007; Semple et al., 2006), insulin also positively regulated in vitro *Adipoq* expression by activating PPARγ, via suppressing FoxO1 activity (Fan et al., 2009). Hence, molecular mechanisms downstream of insulin receptors and CCK receptors could converge at this point to regulate *Adipoq* expression. Our findings are therefore consistent with the close relationship between plasma levels of adiponectin and insulin responsiveness (Cnop et al., 2003), as well as with the hypoadiponectinaemia observed in obese, insulin-resistant patients (Annuzzi et al., 2010; Weyer et al., 2001). Moreover, postprandial plasma levels of CCK are lower in patients with Type 2 diabetes, than in control subjects (Rushakoff et al., 1993), proving a close relationship between CCK, insulin sensitivity, and adiponectin regulation.

Notably, the loss of effect of CCK-8 in pre-adipocytes lacking expression of insulin receptors (Figure 6a–d) supports the idea that both CCK and insulin may co-operate in modulating adiponectin production by WAT. This concept is further supported by the study showing that sub-threshold doses of insulin potentiate the effect of CCK-8 in promoting *Adipoq* expression in both visceral and subcutaneous WAT. As Akt is a common signalling pathway element for both CCK_2 receptors and insulin receptors, all these findings suggest that Akt might account for the interaction between CCK and insulin.

In conclusion, this study showed that CCK-8 stimulated the synthesis of adiponectin by rat adipocytes. Such an effect depended on the activation of PKB (Akt) and involved PPARγ, suggesting that agonists of CCK_2 receptors could have translational value in the design of new insulin-sensitizing therapies. Further, our findings suggest that the insulin-sensitizing effects of CCK agonists already reported by other authors (Irwin et al., 2012) might be linked to the effect of CCK on adiponectin production.

ACKNOWLEDGEMENTS

This study was supported by Ministerio de Ciencia, Innovación y Universidades (BFU2012-35353 and BFU2016-78556-R), European Regional Development Fund, and Fundación Universitaria San Pablo CEU. A.P. is supported by a grant from Ministerio de Ciencia, Innovación y Universidades, Spain (BES-2013-063773).

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

A.P., B.M. and M.R.-G. designed the research. A.P. and B.M. performed the experiments. A.P., B.M. and M.R.-G. analyzed the data. A.P. and M.R.-G. wrote the manuscript. N.D.O. revised the manuscript.

DECLARATION OF TRANSPARENCY AND SCIENTIFIC RIGOUR

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the *BJP* guidelines for Design & Analysis, Immunoblotting and Immunochemistry, and Animal Experimentation, and as recommended by funding agencies, publishers, and other organizations engaged with supporting research.

ORCID

Adrián Plaza https://orcid.org/0000-0002-5316-5090
Beatriz Merino https://orcid.org/0000-0002-2186-070X
Nuria Del Olmo https://orcid.org/0000-0001-5611-4152
Mariano Ruiz-Gayo https://orcid.org/0000-0002-2805-5076

REFERENCES

Alexander, S. P. H., Christopoulos, A., Davenport, A. P., Kelly, E., Marrion, N. V., Peters, J. A., … CGTP Collaborators. (2017). The Concise Guide to PHARMACOLOGY 2017/18: G protein-coupled receptors. *British Journal of Pharmacology*, *174*, S17–S129.

Alexander, S. P. H., Cidlowski, J. A., Kelly, E., Marrion, N. V., Peters, J. A., Faccenda, E., … CGTP Collaborators. (2017). The Concise Guide to PHARMACOLOGY 2017/18: Nuclear hormone receptors. *British Journal of Pharmacology*, *174*, S208–S224. https://doi.org/10.1111/bph.13880

Alexander, S. P. H., Fabbro, D., Kelly, E., Marrion, N. V., Peters, J. A., Faccenda, E., … CGTP Collaborators. (2017). The Concise Guide to PHARMACOLOGY 2017/18: Enzymes. *British Journal of Pharmacology*, *174*, S272–S359. https://doi.org/10.1111/bph.13877

Annuzzi, G., Bozzetto, L., Patti, L., Santangelo, C., Giacco, R., Di Marino, L., … Rivellese, A. A. (2010). Type 2 diabetes mellitus is characterized by reduced postprandial adiponectin response: A possible link with diabetic postprandial dyslipidemia. *Metabolism-Clinical and Experimental*, *59*(4), 567–574. https://doi.org/10.1016/j.metabol.2009.08.020

Baglioni, S., Cantini, G., Poli, G., Francalanci, M., Squecco, R., Di Franco, A., … Luconi, M. (2012). Functional differences in visceral and subcutaneous fat pads originate from differences in the adipose stem cell. *PLoS ONE*, *7*(5), e36569. https://doi.org/10.1371/journal.pone.0036569

Banga, A., Unal, R., Tripathi, P., Pokrovskaya, I., Owens, R. J., Kern, P. A., & Ranganathan, G. (2009). Adiponectin translation is increased by the PPARγ agonists pioglitazone and ω-3 fatty acids. *American Journal of Physiology-Endocrinology and Metabolism*, *296*(3), E480–E489. https://doi.org/10.1152/ajpendo.90892.2008

Basu, R., Pajvani, U. B., Rizza, R. A., & Scherer, P. E. (2007). Selective downregulation of the high–molecular weight form of adiponectin in hyperinsulinemia and in type 2 diabetes: Differential regulation from nondiabetic subjects. *Diabetes*, *56*(8), 2174–2177. https://doi.org/10.2337/db07-0185

Beglinger, C., & Degen, L. (2004). Fat in the intestine as a regulator of appetite—Role of CCK. *Physiology & Behavior*, *83*(4), 617–621. https://doi.org/10.1016/j.physbeh.2004.07.031

Blüher, M. (2016). Adipose tissue inflammation: A cause or consequence of obesity-related insulin resistance? *Clinical Science*, *130*(18), 1603–1614. https://doi.org/10.1042/CS20160005

Bosco, D. B., Roycik, M. D., Jin, Y., Schwartz, M. A., Lively, T. J., Zorio, D. A., & Sang, Q. A. (2017). A new synthetic matrix metalloproteinase inhibitor reduces human mesenchymal stem cell adipogenesis. *PLoS ONE*, *12*(2), e0172925. https://doi.org/10.1371/journal.pone.0172925

Cano, V., Merino, B., Ezquerra, L., Somoza, B., & Ruiz-Gayo, M. (2008). A cholecystokinin-1 receptor agonist (CCK-8) mediates increased permeability of brain barriers to leptin. *British Journal of Pharmacology*, *154*(5), 1009–1015. https://doi.org/10.1038/bjp.2008.149

Chang, C.-L., Au, L.-C., Huang, S.-W., Fai Kwok, C., Ho, L.-T., & Juan, C.-C. (2010). Insulin up-regulates heme oxygenase-1 expression in 3T3-L1 adipocytes via PI3-kinase-and PKC-dependent pathways and heme oxygenase-1–associated microRNA downregulation. *Endocrinology*, *152*(2), 384–393.

Chang, R., Chen, T., Bock, M., Freidinger, R., Chen, R., Rosegay, A., & Lotti, V. J. (1989). Characterization of the binding of [3H] L-365,260: A new potent and selective brain cholecystokinin (CCK-B) and gastrin receptor antagonist radioligand. *Molecular Pharmacology*, *35*(6), 803–808.

Chen, D., Zhao, C. M., Al–Haider, W., Håkanson, R., Rehfeld, J. F., & Kopin, A. S. (2002). Differentiation of gastric ECL cells is altered in CCK2 receptor–deficient mice. *Gastroenterology*, *123*(2), 577–585. https://doi.org/10.1053/gast.2002.34746

Cnop, M., Havel, P., Utzschneider, K., Carr, D., Sinha, M., Boyko, E., … Kahn, S. E. (2003). Relationship of adiponectin to body fat distribution, insulin sensitivity and plasma lipoproteins: Evidence for independent roles of age and sex. *Diabetologia*, *46*(4), 459–469. https://doi.org/10.1007/s00125-003-1074-z

Curtis, M. J., Alexander, S., Cirino, G., Docherty, J. R., George, C. H., Giembycz, M. A., … Ahluwalia, A. (2018). Experimental design and analysis and their reporting II: updated and simplified guidance for authors and peer reviewers. *British Journal of Pharmacology*, *175*, 987–993. https://doi.org/10.1111/bph.14153

Debas, H. T., Farooq, O., & Grossman, M. I. (1975). Inhibition of gastric emptying is a physiological action of cholecystokinin. *Gastroenterology*, *68*(5), 1211–1217.

Degawa-Yamauchi, M., Moss, K. A., Bovenkerk, J. E., Shankar, S. S., Morrison, C. L., Lelliott, C. J., … Considine, R. V. (2005). Regulation of adiponectin expression in human adipocytes: Effects of adiposity, glucocorticoids, and tumor necrosis factor α. *Obesity Research*, *13*(4), 662–669. https://doi.org/10.1038/oby.2005.74

Dufresne, M., Seva, C., & Fourmy, D. (2006). Cholecystokinin and gastrin receptors. *Physiological Reviews*, *86*(3), 805–847. https://doi.org/10.1152/physrev.00014.2005

Fan, W., Imamura, T., Sonoda, N., Sears, D. D., Patsouris, D., Kim, J. J., & Olefsky, J. M. (2009). FOXO1 transrepresses peroxisome proliferator-activated receptor γ transactivation, coordinating an insulin-induced feed-forward response in adipocytes. *Journal of Biological Chemistry*, *284*(18), 12188–12197. https://doi.org/10.1074/jbc.M808915200

Fang, H., & Judd, R. L. (2018). Adiponectin regulation and function. *Comprehensive Physiology*, *8*(3), 1031–1063.

Fasshauer, M., Klein, J., Neumann, S., Eszlinger, M., & Paschke, R. (2002). Hormonal regulation of adiponectin gene expression in 3T3-L1 adipocytes. *Biochemical and Biophysical Research Communications*, *290*(3), 1084–1089. https://doi.org/10.1006/bbrc.2001.6307

Frühbeck, G., Catalán, V., Rodríguez, A., & Gómez-Ambrosi, J. (2018). Adiponectin-leptin ratio: A promising index to estimate adipose tissue dysfunction. Relation with obesity-associated cardiometabolic risk. *Adipocytes*, *7*(1), 57–62. https://doi.org/10.1080/21623945.2017.1402151

Frühbeck, G., Catalán, V., Rodríguez, A., Ramírez, B., Becerril, S., Portincasa, P., & Gómez-Ambrosi, J. (2017). Normalization of adiponectin concentrations by leptin replacement in ob/ob mice is accompanied by reductions in systemic oxidative stress and inflammation. *Scientific Reports*, *7*(1), 2752. https://doi.org/10.1038/s41598-017-02848-0

Gavrila, A., Peng, C.-K., Chan, J. L., Mietus, J. E., Goldberger, A. L., & Mantzoros, C. S. (2003). Diurnal and ultradian dynamics of serum adiponectin in healthy men: Comparison with leptin, circulating soluble leptin receptor, and cortisol patterns. *The Journal of Clinical Endocrinology & Metabolism*, *88*(6), 2838–2843. https://doi.org/10.1210/jc.2002-021721

Gil-Ortega, M., Garidou, L., Barreau, C., Maumus, M., Breasson, L., Tavernier, G., … Sengenès, C. (2013). Native adipose stromal cells egress from adipose tissue in vivo: Evidence during lymph node activation. *Stem Cells*, *31*(7), 1309–1320. https://doi.org/10.1002/stem.1375

Harding, S. D., Sharman, J. L., Faccenda, E., Southan, C., Pawson, A. J., Ireland, S., … NC-IUPHAR. (2017). The IUPHAR/BPS Guide to PHARMACOLOGY in 2018: Updates and expansion to encompass the new guide to IMMUNOPHARMACOLOGY. *Nucleic Acids Research*, *46*(D1), D1091–D1106.

Havel, P. J., Kasim-Karakas, S., Mueller, W., Johnson, P. R., Gingerich, R. L., & Stern, J. S. (1996). Relationship of plasma leptin to plasma insulin and adiposity in normal weight and overweight women: Effects of dietary fat content and sustained weight loss. *The Journal of Clinical Endocrinology & Metabolism*, *81*(12), 4406–4413.

Höcker, M. (2004). Molecular mechanisms of gastrin-dependent gene regulation. *Annals of the new York Academy of Sciences*, *1014*(1), 97–109.

Irwin, N., Frizelle, P., Montgomery, I., Moffett, R., O'Harte, F., & Flatt, P. (2012). Beneficial effects of the novel cholecystokinin agonist (pGlu-Gln)-CCK-8 in mouse models of obesity/diabetes. *Diabetologia*, *55*(10), 2747–2758. https://doi.org/10.1007/s00125-012-2654-6

Irwin, N., Montgomery, I., O'harte, F., Frizelle, P., & Flatt, P. (2013). Comparison of the independent and combined metabolic effects of subchronic modulation of CCK and GIP receptor action in obesity-related diabetes. *International Journal of Obesity*, *37*(8), 1058–1063. https://doi.org/10.1038/ijo.2012.179

Izzo, R. S., Brugge, W. R., & Praissman, M. (1984). Immunoreactive cholecystokinin in human and rat plasma: Correlation of pancreatic secretion in response to CCK. *Regulatory Peptides*, *9*(1–2), 21–34. https://doi.org/10.1016/0167-0115(84)90004-1

Kadowaki, T., Yamauchi, T., Kubota, N., Hara, K., Ueki, K., & Tobe, K. (2006). Adiponectin and adiponectin receptors in insulin resistance, diabetes, and the metabolic syndrome. *The Journal of Clinical Investigation*, *116*(7), 1784–1792. https://doi.org/10.1172/JCI29126

Kantartzis, K., Fritsche, A., Tschritter, O., Thamer, C., Haap, M., Schäfer, S., … Stefan, N. (2005). The association between plasma adiponectin and insulin sensitivity in humans depends on obesity. *Obesity*, *13*(10), 1683–1691. https://doi.org/10.1038/oby.2005.206

Kilkenny, C., Browne, W., Cuthill, I. C., Emerson, M., & Altman, D. G. (2010). Animal research: Reporting *in vivo* experiments: The ARRIVE guidelines. *British Journal of Pharmacology*, *160*, 1577–1579.

King, A., Yang, Q., Huesman, S., Rider, T., & Lo, C. C. (2015). Lipid transport in cholecystokinin knockout mice. *Physiology & Behavior*, *151*, 198–206. https://doi.org/10.1016/j.physbeh.2015.07.009

Kraly, F. S. (1981). A diurnal variation in the satiating potency of cholecystokinin in the rat. *Appetite*, *2*(3), 177–191. https://doi.org/10.1016/S0195-6663(81)80041-4

Kumada, M., Kihara, S., Sumitsuji, S., Kawamoto, T., Matsumoto, S., Ouchi, N., … Matsuzawa, Y. (2003). Association of hypoadiponectinemia with coronary artery disease in men. *Arteriosclerosis, Thrombosis, and Vascular Biology*, *23*(1), 85–89. https://doi.org/10.1161/01.ATV.0000048856.22331.50

Lafontan, M., & Girard, J. (2008). Impact of visceral adipose tissue on liver metabolism: Part I: Heterogeneity of adipose tissue and functional properties of visceral adipose tissue. *Diabetes & Metabolism*, *34*(4), 317–327. https://doi.org/10.1016/j.diabet.2008.04.001

Lee, B., & Shao, J. (2014). Adiponectin and energy homeostasis. *Reviews in Endocrine and Metabolic Disorders*, *15*(2), 149–156. https://doi.org/10.1007/s11154-013-9283-3

Lee, G., Elwood, F., McNally, J., Weiszmann, J., Lindstrom, M., Amaral, K., … Li, Y. (2002). T0070907, a selective ligand for peroxisome proliferator-activated receptor γ, functions as an antagonist of biochemical and cellular activities. *Journal of Biological Chemistry*, *277*(22), 19649–19657. https://doi.org/10.1074/jbc.M200743200

Li, J., Xue, Y.-M., Zhu, B., Pan, Y.-H., Zhang, Y., Wang, C., & Li, Y. (2018). Rosiglitazone elicits an adiponectin-mediated insulin-sensitizing action at the adipose tissue-liver axis in Otsuka Long-Evans Tokushima Fatty rats. *Journal of Diabetes Research*, *2018*, 1–12. https://doi.org/10.1155/2018/4627842

Livak, K. J., & Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods*, *25*(4), 402–408. https://doi.org/10.1006/meth.2001.1262

Maeda, N., Takahashi, M., Funahashi, T., Kihara, S., Nishizawa, H., Kishida, K., … Matsuzawa, Y. (2001). PPARγ ligands increase expression and plasma concentrations of adiponectin, an adipose-derived protein. *Diabetes*, *50*(9), 2094–2099. https://doi.org/10.2337/diabetes.50.9.2094

Merino, B., Cano, V., Guzman, R., Somoza, B., & Ruiz-Gayo, M. (2008). Leptin-mediated hypothalamic pathway of cholecystokinin (CCK-8) to regulate body weight in free-feeding rats. *Endocrinology*, *149*(4), 1994–2000. https://doi.org/10.1210/en.2007-1286

Merino, B., Somoza, B., Ruiz-Gayo, M., & Cano, V. (2008). Circadian rhythm drives the responsiveness of leptin-mediated hypothalamic pathway of cholecystokinin-8. *Neuroscience Letters*, *442*(2), 165–168. https://doi.org/10.1016/j.neulet.2008.07.009

Miller, L. J., & Gao, F. (2008). Structural basis of cholecystokinin receptor binding and regulation. *Pharmacology & Therapeutics*, *119*(1), 83–95. https://doi.org/10.1016/j.pharmthera.2008.05.001

Pathak, V., Flatt, P. R., & Irwin, N. (2018). Cholecystokinin (CCK) and related adjunct peptide therapies for the treatment of obesity and type 2 diabetes. *Peptides*, *100*, 229–235.

Pereira, R. I., & Draznin, B. (2005). Inhibition of the phosphatidylinositol 3′-kinase signaling pathway leads to decreased insulin-stimulated adiponectin secretion from 3T3-L1 adipocytes. *Metabolism*, *54*(12), 1636–1643. https://doi.org/10.1016/j.metabol.2005.07.002

Plaza, A., Merino, B., Cano, V., Domínguez, G., Pérez-Castells, J., Fernández-Alfonso, M. S., … Ruiz-Gayo, M. (2018). Cholecystokinin is involved in triglyceride fatty acid uptake by rat adipose tissue. *Journal of Endocrinology*, *236*(3), 137–150. https://doi.org/10.1530/JOE-17-0580

Plaza, A., Merino, B., Sánchez-Pernaute, A., Torres-García, A. J., Rubio-Herrera, M. A., & Ruiz-Gayo, M. (2018). Expression analysis of a cholecystokinin system in human and rat white adipose tissue. *Life Sciences*, *206*, 98–105. https://doi.org/10.1016/j.lfs.2018.05.036

Poncelet, M., Arnone, M., Heaulme, M., Gonalons, N., Gueudet, C., Santucci, V., … Soubrié, P. (1993). Neurobehavioural effects of SR 27897, a selective cholecystokinin type A (CCK-A) receptor antagonist. *Naunyn-Schmiedeberg's Archives of Pharmacology*, *348*(1), 102–107. https://doi.org/10.1007/BF00168544

Reinehr, T., Roth, C., Menke, T., & Andler, W. (2004). Adiponectin before and after weight loss in obese children. *The Journal of Clinical Endocrinology & Metabolism*, *89*(8), 3790–3794. https://doi.org/10.1210/jc.2003-031925

Rushakoff, R. A., Goldfine, I., Beccaria, L. J., Mathur, A., Brand, R. J., & Liddle, R. A. (1993). Reduced postprandial cholecystokinin (CCK) secretion in patients with noninsulin-dependent diabetes mellitus: Evidence for a role for CCK in regulating postprandial hyperglycemia. *The Journal of Clinical Endocrinology & Metabolism*, *76*(2), 489–493.

Scheer, F., Chan, J., Fargnoli, J., Chamberland, J., Arampatzi, K., Shea, S., … Mantzoros, C. S. (2010). Day/night variations of high-molecular-weight adiponectin and lipocalin-2 in healthy men studied under fed and fasted conditions. *Diabetologia*, *53*(11), 2401–2405. https://doi.org/10.1007/s00125-010-1869-7

Semple, R., Soos, M., Luan, J., Mitchell, C., Wilson, J., Gurnell, M., … O'Rahilly, S. (2006). Elevated plasma adiponectin in humans with

genetically defective insulin receptors. *The Journal of Clinical Endocrinology & Metabolism*, *91*(8), 3219–3223. https://doi.org/10.1210/jc.2006-0166

Shao, X., Wang, M., Wei, X., Deng, S., Fu, N., Peng, Q., … Lin, Y. (2016). Peroxisome proliferator-activated receptor-γ: master regulator of adipogenesis and obesity. *Current Stem Cell Research & Therapy*, *11*(3), 282–289. https://doi.org/10.2174/1574888X10666150528144905

Singer, M. (1987). Pancreatic secretory response to intestinal stimulants: A review. *Scandinavian Journal of Gastroenterology*, *22*(sup139), 1–13. https://doi.org/10.3109/00365528709089768

Singh, P., Sharma, P., Sahakyan, K. R., Davison, D. E., Sert-Kuniyoshi, F. H., Romero-Corral, A., … Somers, V. K. (2016). Differential effects of leptin on adiponectin expression with weight gain versus obesity. *International Journal of Obesity*, *40*(2), 266–274. https://doi.org/10.1038/ijo.2015.181

Stern, J. H., Rutkowski, J. M., & Scherer, P. E. (2016). Adiponectin, leptin, and fatty acids in the maintenance of metabolic homeostasis through adipose tissue crosstalk. *Cell Metabolism*, *23*(5), 770–784. https://doi.org/10.1016/j.cmet.2016.04.011

Tripathy, D., Daniele, G., Fiorentino, T. V., Perez-Cadena, Z., Chavez-Velasquez, A., Kamath, S., … Folli, F. (2013). Pioglitazone improves glucose metabolism and modulates skeletal muscle TIMP-3–TACE dyad in type 2 diabetes mellitus: A randomised, double-blind, placebo-controlled, mechanistic study. *Diabetologia*, *56*(10), 2153–2163. https://doi.org/10.1007/s00125-013-2976-z

Tzivion, G., Dobson, M., & Ramakrishnan, G. (2011). FoxO transcription factors; regulation by AKT and 14-3-3 proteins. *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research*, *1813*(11), 1938–1945. https://doi.org/10.1016/j.bbamcr.2011.06.002

Weyer, C., Funahashi, T., Tanaka, S., Hotta, K., Matsuzawa, Y., Pratley, R. E., & Tataranni, P. A. (2001). Hypoadiponectinemia in obesity and type 2 diabetes: Close association with insulin resistance and hyperinsulinemia. *The Journal of Clinical Endocrinology & Metabolism*, *86*(5), 1930–1935. https://doi.org/10.1210/jcem.86.5.7463

Yamauchi, T., Kamon, J., Waki, H., Terauchi, Y., Kubota, N., Hara, K., … Kadowaki, T. (2001). The fat-derived hormone adiponectin reverses insulin resistance associated with both lipoatrophy and obesity. *Nature Medicine*, *7*(8), 941–946. https://doi.org/10.1038/90984

Yatagai, T., Nagasaka, S., Taniguchi, A., Fukushima, M., Nakamura, T., Kuroe, A., … Ishibashi, S. (2003). Hypoadiponectinemia is associated with visceral fat accumulation and insulin resistance in Japanese men with type 2 diabetes mellitus. *Metabolism-Clinical and Experimental*, *52*(10), 1274–1278. https://doi.org/10.1016/S0026-0495(03)00195-1

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

How to cite this article: Plaza A, Merino B, Del Olmo N, Ruiz-Gayo M. The cholecystokinin receptor agonist, CCK-8, induces adiponectin production in rat white adipose tissue. *Br J Pharmacol*. 2019;176:2678–2690. https://doi.org/10.1111/bph.14690