Archives of Toxicology (2018) 92:1717–1728
https://doi.org/10.1007/s00204-018-2174-3

MOLECULAR TOXICOLOGY

New mechanistic insights on the metabolic-disruptor role of chlorpyrifos in apoE mice: a focus on insulin- and leptin-signalling pathways

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Received: 5 December 2017 / Accepted: 31 January 2018 / Published online: 5 February 2018

Abstract

Recently, we have provided evidence, suggesting that mice expressing the human apolipoprotein E3 (apoE3) are more prone to develop an obesity-like phenotype and a diabetic profile when subchronically fed a chlorpyrifos (CPF)-supplemented diet. The aim of the current study was to examine the underlying mechanisms through which CPF alters both insulin- and leptin-signalling pathways in an *APOE*-dependent manner. Both adult apoE3- and E4-targeted replacement and C57BL/6 mice were exposed to CPF at 0 or 2 mg/kg body weight/day through the diet for 8 consecutive weeks. We determined the expression of JAK2, p-JAK2, STAT3, p-STAT3, SOCS3, IRS-1, p-IRS-1, AKT, p-AKT, GSK3β, p-GSK3β, and apoE in the liver, as well as hepatic mRNA levels of *pon1, pon2*, and *pon3*. CPF markedly disrupted both leptin and insulin homeostasis, particularly in apoE3 mice. Indeed, only CPF-fed apoE3 mice exhibited an increased phosphorylation ratio of STAT3, as well as increased total SOCS3 protein levels. Similarly, the exposure to CPF drastically reduced the phosphorylation ratio of both AKT and GSK3β, especially in apoE3 mice. Overall, CPF reduced the expression of the three *pon* genes, principally in C57BL/6 and apoE3 mice. These results provide notable mechanistic insights on the metabolic effects of the pesticide CPF, and attest the increased vulnerability of apoE3 carriers to its metabolic-disruptor role.

Keywords Apolipoprotein E · Insulin · Leptin · Paraoxonase · Chlorpyrifos · Pesticide · Diabetes

Introduction

The dual epidemics of type 2 diabetes (T2D) and obesity has undeniably become an urgent global socio-economic and health problem. According to the International Diabetes Federation (IDF), the number of people suffering from diabetes worldwide was estimated to be 415 million in 2015. Globally, if these trends continue, almost one adult in ten will live with this condition by 2040 (IDF 2017). Traditionally, efforts to prevent and tackle diabetes have focused on addressing some well-known factors (e.g., age, sex, genetics, and lifestyle), which constantly raises the question as to what extent they are able by themselves to account for the global staggering pace of the disease. Accordingly, it has become increasingly evident that environmental exposures to health hazards, including pesticides, may be of critical concern in the global diabetes epidemic (Chevalier and Fénichel 2015; Thayer et al. 2012). While most investigations have traditionally focused on deciphering the contribution of organochlorine (OC) pesticides in triggering T2D and related metabolic

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dysfunctions (Abou-Donia et al. 2006; Aminov et al. 2016; Dirinck et al. 2014; Everett et al. 2017; Grice et al. 2017; Stapleton and Chan 2009; Suarez-Lopez et al. 2015), much less is known about the impact of organophosphate (OP) compounds on these diseases (Montgomery et al. 2008; Slotkin 2011; Starling et al. 2014). Indeed, OP pesticides—and chlorpyrifos (CPF) in particular—have cornered the selling market since the late 1970s; their lower environmental persistence than OC agents and high effectiveness against different insect species are qualities that still make them one of the most widely used pesticides worldwide (Saunders et al. 2012). Although environmental agencies have attempted to restrict CPF use, recent data suggested that its residues are still detectable not only in urban and rural areas (Ccanccapa et al. 2016; Roca et al. 2014), but also in foods intended for human consumption (Chiesa et al. 2016; Nougadère et al. 2012).

All compounds belonging to the OP pesticide family share the same primary mechanism of action: they bind to and strongly inhibit both plasma and brain cholinesterases (ChE) and other esterases (Crow et al. 2012; Estévez et al. 2013). However, it is well established that some OPs, as CPF, act beyond their ability to inhibit ChE and that each substance has a unique inhibitor profile (Quistad et al. 2006; Rohlman et al. 2011). It can, therefore, no longer be assumed that all OPs act alike. As a prerequisite for them to express their toxicity, a cytochrome P-450 (CYP)-mediated oxidative reaction must occur. In the case of CPF, its oxygen analogue CPF-oxon can subsequently be inactivated by a paraoxonase 1 (PON1)-dependent reaction, together with a role of albumin (Sogorb and Vilanova 2010). The PON family of enzymes also includes the little known PON2 and PON3, but only PON1 exerts an OP detoxifying activity. A number of studies have shown that the three proteins modulate oxidative stress and inflammation following an isoform-specific pattern (Furlong et al. 2016). Furthermore, recent data have shed light on the role of PON3 in obesity and related metabolic dysfunctions (Shih et al. 2015).

To date, a considerable number of studies have addressed the neurotoxic effects and behavioural outcomes of CPF exposure. However, only a few investigators have considered the metabolic effects of the pesticide (Lassiter and Brimijoin 2008; Meggs and Brewer 2007; Reygner et al. 2016), while the bulk of the existing research focuses on early-life stages. Some of these investigations have suggested that CPF interferes with hormones essential to homeostatic regulation, such as insulin and leptin (Slotkin et al. 2005), but the exact molecular mechanisms remain unclear.

The field of ecogenetics investigates how certain genetic polymorphisms may represent risk factors for a number of diseases associated with exposure to environmental hazards (Costa 2000). In fact, genetically determined variations in biotransformation enzymes (e.g., CYP or PON1) (Cole et al. 2014; Crane et al. 2012) or target molecules (e.g., ChE) (Lockridge et al. 2016) can modify the individual's response to OPs. However, until recently, the search for other potential genetic risk factors was lacking. Briefly, apolipoprotein E (apoE) is a prominent constituent of plasma and brain lipoproteins that mainly exerts an anti-atherogenic function by interacting with members of the low-density lipoprotein receptor family. Three major *APOE* allelic variants exist in humans (i.e., *ε2, ε3*, and *ε4*) in varying frequencies, and although other mammals express the protein, this genetic polymorphism is peerless in the animal kingdom. Over the last few years, our group has focused on studying the extent to which human *APOE* polymorphisms modulate cognitive processes in the absence of disease. We have simultaneously investigated whether mice expressing one of the three human apoE isoforms respond differently when challenged with toxic agents (Peris-Sampedro et al. 2015a, b, 2016; Reverte et al. 2012, 2013, 2014a, b, 2016). An important finding is that apoE3 mice are more prone to gain excess weight than C57BL/6 (Peris-Sampedro et al. 2015b) and are the only ones to do so relative to both apoE2 and apoE4 mice (Peris-Sampedro et al. 2015a) when subchronically fed a diet supplemented with CPF during adulthood. Furthermore, being a carrier of the *ε3* allele resulted in exacerbated plasma leptin and insulin levels, as well as higher HOMA-IR values (Peris-Sampedro et al. 2015b).

Based on our previous results, the present study was aimed at unravelling the potential underlying mechanisms through which the pesticide alters both insulin- and leptin-sensing pathways in apoE3 mice. To that end, we assessed the impact of dietary CPF and *APOE* genotype on (1) the JAK2/STAT3/SOCS3 signalling pathway, as the major pathway of leptin signalling; (2) insulin signalling through IRS-1 and the AKT/GSK3β signalling pathway, which plays a critical role in glucose homeostasis; and (3) the hepatic expression of *pon* polymorphisms.

Materials and methods

Animals

Adult apoE-targeted replacement (TR), homozygous for the human *ε3* and *ε4* alleles (Taconic Europe, Lille Skensved, Denmark), and C57BL/6 (Charles River France, L'Arbresle, France) male mice were used (7 months, $n=24$). The apoE-TR mouse model was designed to express human apoE under the control of the endogenous murine promoter (Sullivan et al. 1997), thereby enabling the expression of the human protein at physiologically regulated levels. Animals were housed under a 12-h light/dark cycle (lights off at 8 pm) in the standard environmentally controlled conditions (22 ± 2 °C, $50 \pm 10\%$ humidity). They had free access to

food before the experiment started, and were fed a standard rodent chow (Panlab, Barcelona, Spain), unless otherwise indicated. Water was available ad libitum all times.

All procedures were approved by the Animal Care and Use Committee of the Rovira i Virgili University (Tarragona, Spain) (ethics permit: 0288GC), and were conducted in compliance with the Spanish Royal Decree 53/2013 on the protection of experimental animals, and the European Communities Council Directive (86/609/EEC). All efforts were made to reduce both animal stress and usage.

Chemicals, treatment, and experimental design

CPF [*O,O*-diethyl *O*-(3,5,6-trichloropyridin-2-yl) phosphorothioate, purity 99.5%] was provided by Sigma-Aldrich (Seelze, Germany). The CPF-supplemented diet was obtained as previously described (Basaure et al. 2017; Peris-Sampedro et al. 2015a, b, 2016). Briefly, the standard rodent chow was supplemented with 20 mg CPF/kg chow. Given the feeding conditions (i.e., 3 g/mouse/day), this processed diet was intended to deliver approximately 2 mg CPF/kg body weight/day. As reported earlier (Peris-Sampedro et al. 2015a, b, 2016), and this dose induces a moderate inhibition of plasma cholinesterase without signs of acute toxicity.

The six experimental groups were as follows ($n = 4$/group): control C57BL/6, CPF-fed C57BL/6, control apoE3, CPF-fed apoE3, control apoE4, and CPF-fed apoE4. Animals were provided with 3 g/mouse/day of either a standard or a CPF-supplemented diet for 8 consecutive weeks. At the end of the treatment period, mice were subjected to a 3-h fast before being deeply anesthetized with carbon dioxide and euthanized. Blood was obtained by cardiac puncture, which was immediately centrifuged to obtain plasma, which was ultimately stored at − 80 °C and thawed once for final analysis. After the blood draw, the median lobe of the liver was removed, dissected, and stored in RNA*later*® (Sigma–Aldrich, Seelze, Germany) at − 80 °C for subsequent western blot analysis and gene expression.

Enzyme activity assessment

Plasma ChE activity was randomly assessed in six mice (controls = 3, CPF-fed = 3) as an indicator of the acute systemic effect of the pesticide (Eaton et al. 2008; Peris-Sampedro et al. 2015a, 2016). Enzymatic assay procedures and a detailed description of the sample processing are available elsewhere (Basaure et al. 2017; Peris-Sampedro et al. 2015a, b, 2016; Salazar et al. 2011). Briefly, enzyme activity was determined spectrophotometrically using the Ellman method (Ellman et al. 1961), and was further calculated relative to the protein content of the sample. The enzyme activity of the exposed animals was estimated based on that of the control mice, and represented as a percentage.

Western blot analysis

All the antibodies used in this study were supplied by Cell-Signalling Technology (Cell-Signalling Technology, New England Biolabs, Beverly, MA, USA) unless otherwise stated. A sample buffer [0.5 M Tris–HCl pH 6.8, 10% glycerol, 2% (wt/vol) SDS, 5% (vol/vol) 2-β-mercaptoethanol, 0.05% bromophenol blue] was added to aliquots of each sample containing 30 µg of protein. The mixture was boiled at 95–100 °C for 5 min to ensure denaturation. The samples were then separated by electrophoresis on 12% acrylamide gels. The proteins were subsequently transferred to Immobilon-P PVDF sheets (Millipore Corp, Bedford, MA, USA) using a transblot apparatus (Bio-Rad, Madrid, Spain). The membranes were blocked for 1 h with a solution containing 5% of skim milk dissolved in TBS-T buffer (50 mM Tris, 1.5% NaCl, 0.05% Tween 20, pH 7.5). The blots were then incubated overnight with primary monoclonal antibodies against AKT, AKT phosphorylated on Ser473, apoE (Abcam, Cambridge, UK), GSK3β, GSK3β phosphorylated on Ser9, IRS-1, IRS-1 phosphorylated on Tyr608 (Millipore Ltd., Wembley, UK), JAK2, JAK2 phosphorylated on Y1007/Y1008, STAT3, STAT3 phosphorylated on Y705, SOCS3, or β-actin (Sigma, St. Louis, MO, USA). Afterwards, the blots were thoroughly washed in TBS-T buffer and incubated for 1 h with a peroxidase-conjugated immunoglobulin G antibody. Immunoreactive proteins were visualized using an Immun-Star Chemiluminescence Kit (Bio-Rad, Madrid, Spain), and the images were ultimately acquired and semi-quantified with Chemidoc™ Imaging System (Bio-rad, Madrid, Spain) (more details in Blanco et al. 2017). We periodically monitored the protein load via the immunodetection of β-actin.

Gene expression

All the products intended for RNA isolation, complementary DNA (cDNA) synthesis, and real-time reverse transcription PCR (RT-PCR) assays were purchased from Qiagen (Qiagen Inc., Hilden, Germany), unless otherwise specified.

RNA isolation and complementary DNA synthesis

Total RNA was extracted from the liver samples with an RNeasy Kit according to the manufacturer's instructions. Briefly, the samples were first lysed and homogenized with highly denaturing guanidine–thiocyanate-containing buffer, which immediately inactivates RNases to ensure purification of intact RNA. After, ethanol was added to provide appropriate binding conditions, and each sample was then applied to an RNeasy Mini spin column, where the total RNA binds to the membrane and contaminants can be efficiently washed away. At the end of the procedure, we obtained high-quality

RNA that was eluted in 100 µl water. Both RNA abundance and purity were assessed spectrophotometrically with NanoDrop technology (i.e., measurement at 260 nm and assessment of the OD_{260}/OD_{280} ratio, respectively), while RNA integrity was tested by electrophoresis on a 1%-denaturing agarose gel. The first strand of cDNA was reverse-transcribed from 1 µg of total RNA from each sample using a QuantiTect Reverse Transcription Kit, following the manufacturer's protocol. To confirm that the samples were free of genomic DNA, we also carried out an identical reaction in the absence of reverse transcriptase. The subsequent cDNA was amplified by PCR with a Dream Taq Hot Start PCR Master Mix kit (Thermo Scientific, Waltham, MA, USA), as recommended by the supplier. We used the mouse-specific primer sequences for *pon1, pon2, pon3*, and *gadph* (Table 1). The PCR products were separated on a 1% agarose gel, and only specific bands were detected. The non-reactivity of the primers with contaminant genomic DNA was tested by including controls that omitted reverse transcriptase from the cDNA synthesis reaction.

Real-time reverse transcription PCR assays

We performed a quantitative RT-PCR assay for *pon1, pon2, pon3*, and *gadph* with a QuantiTect SYBR Green PCR kit in a Rotor-Gene Q Real-Time PCR cycler, according to the manufacturer's protocol. The thermal cycling consisted of a 2-min initial step at 50 °C, followed by a 15-min polymerase activation step at 95 °C, and a cycling step with the following conditions: 40 cycles of denaturing at 95 °C for 15 s, annealing at 60 °C for 30 s, and extension at 72 °C for 30 s. As oligonucleotides of varying lengths produce dissociation peaks at different melting temperatures, the PCR products were analysed at the end of the PCR cycles using a heat dissociation protocol to confirm that a single PCR product was detected by SYBR Green dye. Fluorescence data were acquired during the 72 °C step. The threshold cycle (Ct) was calculated during the early cycles of amplification using the Rotor-Gene Q 2.0 software to identify significant fluorescence signals above noise (Blanco et al. 2012, 2013, 2017). Relative changes in transcript levels were normalized to the RNA levels of *gadph* according to the $2-\Delta\Delta C_t$ method.

Statistics

Data were processed using the SPSS statistical package (version 23.0). We used one-way analysis of variance (ANOVA) to establish the effects of dietary CPF on plasma ChE activity. We performed a Pearson correlation analysis to assess the relationship between the total and the phosphorylated levels of IRS-1 protein. All the other parameters were analysed by two-way ANOVA (treatment, genotype), and further one-way ANOVA (treatment), if necessary. Tukey's post-hoc comparisons were used when appropriate. Statistical significance was set at $p < 0.05$, and the results are reported as mean values ± SD.

Results

ChE activity and apoE protein levels

As in the previous studies (Peris-Sampedro et al. 2015a, b), mice subjected to an 8-week dietary exposure to 2 mg/kg body weight/day CPF did not show any apparent sign of cholinergic toxicity during the treatment period. Relative to the controls, the plasma ChE activity of CPF-exposed mice dropped to 17.76%. On the other hand, neither the treatment nor the genotype affected apoE levels in the liver (Fig. 1).

Effects of CPF and *APOE* genotype on both leptin- and insulin-signalling pathways

The JAK2/STAT3/SOCS3-signalling pathway

In response to leptin, JAK2 phosphorylates the leptin receptor (LEPRb) on Tyr1138, thereby promoting its activation and the subsequent recruitment of the SH2 domain of STAT3. STAT3 is subsequently phosphorylated by the complex LEPRb-JAK2, resulting in dimerization and nuclear translocation. Once in the nucleus, STAT3

Table 1 Primers used for RT-PCR analysis

Gene	Accession number	Orientation	Primer sequence
pon1	NM_011134	Forward	5′-TTGCTGCACTTGTCCATCCTC-3′
		Reverse	5′-ACTGCTGGCTCCTTCTTGTTC-3′
pon2	NM_183308	Forward	5′-ATGGTGGCTCTGAGTTTG-3′
		Reverse	5′-TCCTCAGCTCCAGTTTCGAT-3′
pon3	NM_173006	Forward	5′- GATCTGAATGAGCAAAACCCAGAGGC-3′
		Reverse	5′- GAGTCCATGTTGGGGTGATTCACGAC-3′
gadph	NM_001289726	Forward	5′-ACAACTTTGGCATTGTGGAA-3′
		Reverse	5′-GATGCAGGGATGATGTTCTG-3′

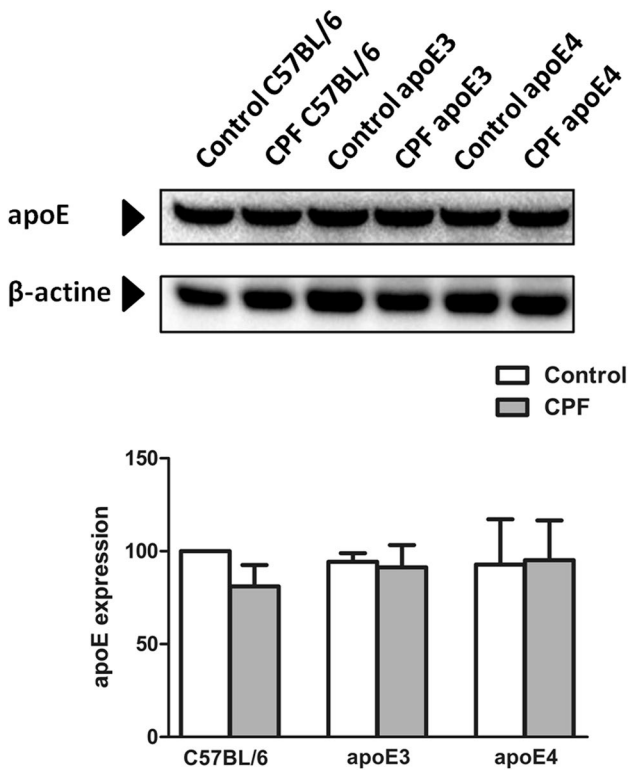

Fig. 1 Hepatic expression of apoE protein in apoE3, apoE4, and C57BL/6 adult male mice upon an 8-week dietary exposure to CPF. The upper panel provides the images of a representative western blot experiment

dimers act as a transcription factor regulating the expression of target genes such as SOCS3, which ultimately acts as a negative feedback regulator antagonizing LEPRb signalling.

Figure 2 provides an overview of the genotype-dependent effects of dietary CPF on the JAK2/STAT3 pathway and on subsequent total SOCS3 levels.

Overall, CPF exposure increased, while the genotype influenced both the p-STAT3/total STAT3 ratio (treatment: $F_{1,20} = 5.250$, $p = 0.037$; genotype: $F_{2,20} = 8.723$, $p = 0.003$) and total SOCS3 levels (treatment: $F_{1,22} = 4.558$, $p = 0.048$; genotype: $F_{2,22} = 6.706$, $p = 0.007$). Specifically, apoE3 mice displayed the highest phosphorylation ratio of STAT3 (apoE3 vs apoE4: $p = 0.027$; apoE3 vs C57BL/6: $p = 0.008$), and exhibited increased SOCS3 levels relative to both apoE4 ($p = 0.052$) and C57BL/6 ($p = 0.016$) mice. We also observed a genotype × treatment interaction for these two parameters (STAT3: $F_{2,20} = 5.424$, $p = 0.017$; SOCS3: $F_{2,22} = 4.151$, $p = 0.034$). Reanalyses showed, albeit not significantly, that CPF exposure increased the phosphorylation of STAT3 in apoE3 ($F_{1,6} = 5.745$, $p = 0.062$) (Fig. 2b). Similarly, only the apoE3 individuals that were exposed to the pesticide exhibited increased SOCS3 levels ($F_{1,6} = 13.354$, $p = 0.015$) (Fig. 2c).

On the other hand, the treatment significantly increased the p-JAK2/total JAK2 ratio, regardless of the genotype ($F_{1,22} = 12.487$, $p = 0.003$) (Fig. 2a).

Insulin-signalling pathway: p-IRS-1/total IRS-1, p-AKT/total AKT and p-GSK3β/total GSK3β ratios

The binding of insulin to the α subunit of the insulin receptor (IR) results in the autophosphorylation of a number of tyrosine residues present in the β subunit, which in turn promotes the phosphorylation of IRS-1 protein. Active IRS-1 protein triggers the activation of a signalling cascade through PI3K, resulting in the activation of AKT by phosphorylation. Once active, AKT leads to the phosphorylation and subsequent inactivation of GSK3β, which ultimately results in glycogen synthesis.

The effects of CPF on insulin-signalling pathways are set out, as shown in Fig. 3. Neither the treatment nor the genotype affected the phosphorylation of IRS-1. Interestingly, the changes in p-IRS-1 levels were positively and strongly correlated to those of the total protein ($r = 0.813$, $p < 0.001$). Indeed, although we did not found any significant result, CPF exposure tended to influence both the total and the phosphorylated forms of IRS-1 in a genotype-dependent pattern. In turn, while CPF-treated apoE3 mice displayed lower levels of these two forms, both apoE4 and C57BL/6 individuals did so conversely (Fig. 3a).

We also observed a significant genotype × treatment interaction for the ratios of phosphorylation of both AKT ($F_{2,22} = 6.107$, $p = 0.010$) and GSK3β ($F_{2,22} = 9.816$, $p = 0.001$) proteins. The CPF-exposed apoE3 mice were indeed the only ones that displayed a sharp decline in the phosphorylation of AKT ($F_{1,6} = 21.786$, $p = 0.005$) (Fig. 3b) and GSK3β ($F_{1,6} = 33.533$, $p = 0.002$) (Fig. 3c) relative to their control peers. In line with this, we found that the treatment led to a marked increase in the phosphorylation of GSK3β in C57BL/6 mice ($F_{1,7} = 7.015$, $p = 0.038$) (Fig. 3c).

Impact of CPF and *APOE* genotype on the expression of *pon1*, *pon2*, and *pon3*

PON enzymes enhance the liver's capacity for the antioxidative and anti-inflammatory defence. The hydrolytic capacity of PON1 to detoxify oxygen analogues of certain OPs was discovered more than six decades ago. While the scientific literature about genetic and developmental variability of PON1 has increased significantly, far too little attention has been paid to the other most common PON enzymes, namely, PON2 and PON3.

The relative expression of the three *pon* genes in the liver is shown in Fig. 4. Both the treatment and the genotype were found to modulate the expression of *pon1* (treatment: $F_{1,20} = 30.862$, $p < 0.001$; genotype: $F_{2,20} = 11.903$, $p = 0.001$),

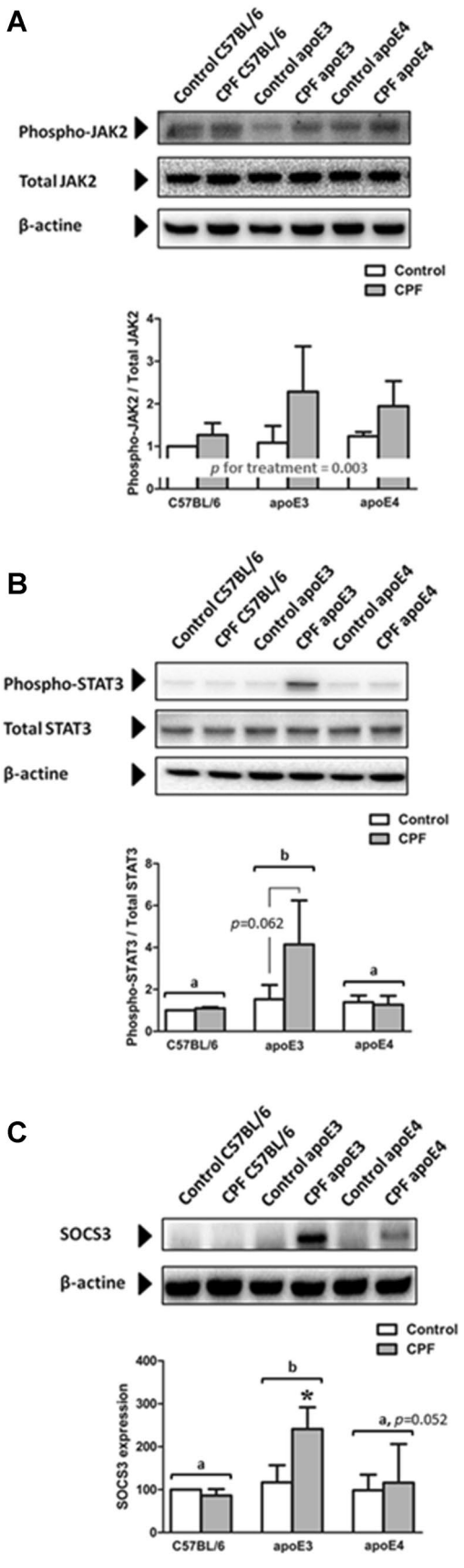

Fig. 2 Impact of an 8-week dietary exposure to CPF on leptin-signalling pathway in apoE3, apoE4 and C57BL/6 adult male mice. The relative levels of hepatic protein expression of **a** p-JAK2/total JAK2 and **b** p-STAT3/total STAT3 ratios, as well as the expression of **c** total SOCS3 protein are depicted. The upper panel provides the images of a representative western blot experiment. Groups showing different letters (**a, b**) differ significantly from each other at $p < 0.05$. The symbol in the lower panel indicates effects of the treatment within each group at $p < 0.05$ (*)

pon2 (treatment: $F_{1,19} = 11.937$, $p = 0.004$; genotype: $F_{2,19} = 24.935$, $p < 0.001$), and *pon3* (treatment: $F_{1,18} = 30.105$, $p < 0.001$; genotype: $F_{2,18} = 11.945$, $p = 0.001$). Specifically, the 8-week dietary exposure to CPF led to an overall reduction in the expression of the three genes. Moreover, C57BL/6 mice were the ones that most expressed both *pon1* (C57BL/6 vs apoE3: $p = 0.001$, C57BL/6 vs apoE4: $p = 0.005$) and *pon3* (C57BL/6 vs apoE3: $p = 0.001$, C57BL/6 vs apoE4: $p = 0.028$). Conversely, *ε3* carriers expressed *pon2* to a lesser extent than the other two genotypes (apoE3 vs C57BL/6: $p = 0.001$, apoE3 vs apoE4: $p < 0.001$). We also noted a significant genotype × treatment interaction for *pon1* ($F_{2,20} = 5.424$, $p = 0.017$) and *pon3* ($F_{2,18} = 6.386$, $p = 0.012$). Reanalysis showed that the pesticide significantly reduced the expression of *pon1* (Fig. 4a) and *pon3* (Fig. 4c) in C57BL/6 ($F_{1,5} = 14.749$, $p = 0.018$ and $F_{1,5} = 15.821$, $p = 0.016$, respectively) and apoE3 mice ($F_{1,6} = 8.108$, $p = 0.036$ and $F_{1,6} = 9.074$, $p = 0.030$, respectively). Similarly, CPF-fed C57BL/6 mice expressed less *pon2* than their control counterparts did ($F_{1,6} = 11.546$, $p = 0.019$) (Fig. 4b).

Discussion

In this study, we sought to shed light on the molecular mechanisms able to explain the *APOE*-dependent metabolic-disruptor role of CPF. We investigated both leptin- and insulin-signalling pathways, as well as the hepatic expression of *pon1, pon2* and *pon3* following an 8-week dietary exposure to the pesticide in apoE3, apoE4, and C57BL/6 adult male mice. The results indicate that repeated dietary doses of CPF, devoid of signs of cholinergic toxicity, notably disrupted leptin and insulin homeostasis, as well as broadly reduced the expression of the three *pon* genes. Furthermore, and in line with the results of our previous studies (Peris-Sampedro et al. 2015a, b), apoE3 mice were the most affected. Indeed, only CPF-fed apoE3 mice exhibited increased phosphorylation ratio of STAT3, as well as increased total SOCS3 protein levels. Likewise, exposure to CPF drastically reduced the phosphorylation ratio of both AKT and GSK3β, particularly in apoE3 mice. Meanwhile, C57BL/6 mice showed the highest expression of *pon1* and *pon3*, while mice homozygous for the *ε3* allele expressed the least *pon2* basally. Overall, the expression of the three

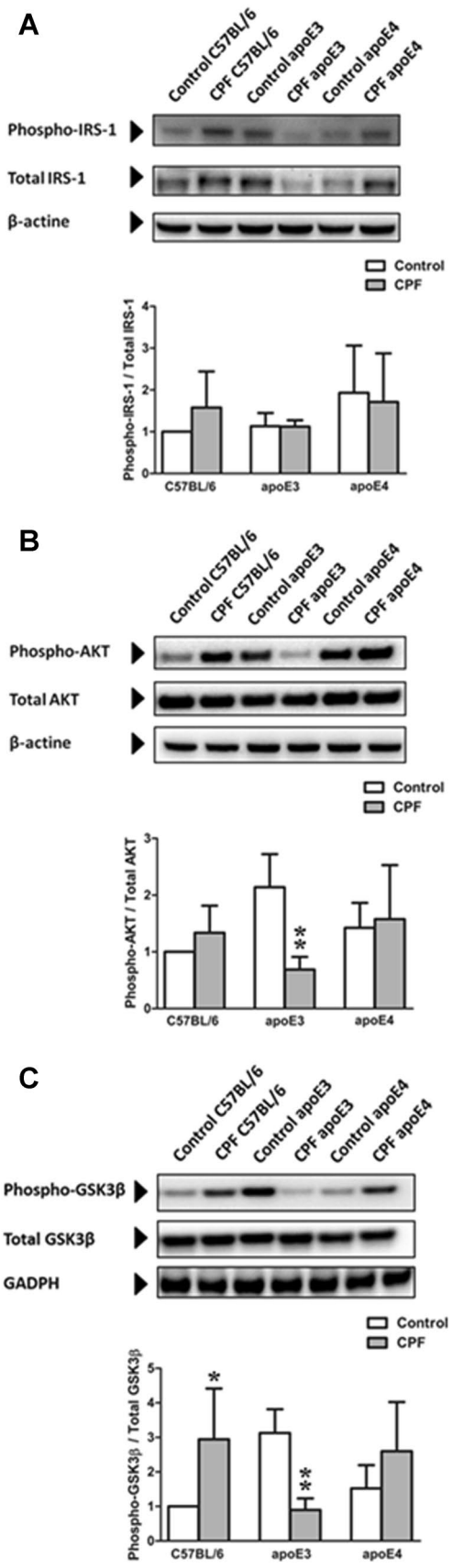

Fig. 3 Impact of an 8-week dietary exposure to CPF on insulin-signalling pathway in apoE3, apoE4 and C57BL/6 adult male mice. The relative levels of hepatic protein expression of: **a** p-IRS-1/total IRS-1, **b** p-AKT/total AKT, and **c** p-GSK3β/total GSK3β ratios are illustrated. The upper panel provides the images of a representative western blot experiment. Symbols in the lower panel indicate effects of the treatment within each group at $p < 0.05$ (*) and $p < 0.01$ (**)

genes dropped after the exposure, being this decline more pronounced in C57BL/6 and apoE3 mice.

Similar to that we have previously found (Peris-Sampedro et al. 2015a, b, 2016), an 8-week exposure to 2 mg CPF/kg body weight/day induced an 82.24% asymptomatic inhibition of plasma ChE activity. This enzyme is mainly synthetized in the liver and secreted in plasma. Although its specific physiological function has not yet been elucidated, it is well-established that plasma ChE exerts a protective role against several exogenous substances (e.g., cocaine, acetylsalicylic acid, and procaine), including OPs (Lockridge et al. 2016). Indeed, plasma ChE is crucial to cushion the CPF-related neurotoxic effects, since it prevents or at least minimizes CPF-oxon binding to its primary brain target (i.e., acetylcholinesterase) (Costa 2006). In this sense, some authors have reported significant inhibitions of plasma ChE, while brain ChE activity remained unchanged in rodents exposed to either low or moderate doses of CPF (Carr et al. 2014; Ricceri et al. 2006). Therefore, we assume that CPF-fed mice were free from any significant central cholinergic toxicity.

To the best of our knowledge, this study is the first to reveal not only that CPF disrupts the JAK2/STAT3 pathway in rodents, but also that it does so in an *APOE*-dependent manner. We have shown that in overall terms, CPF exposure increased the phosphorylation ratio of JAK2 and STAT3, as well as total SOCS3 protein levels. However, these differences were mainly due to a genotype effect. In fact, only apoE3 mice displayed higher p-JAK2 and STAT3 protein levels when challenged with CPF. We recently demonstrated that mice carrying the *ε3* allele were more likely to gain excess weight than C57BL/6 (Peris-Sampedro et al. 2015b), apoE2 and apoE4 mice (Peris-Sampedro et al. 2015a) after subchronic exposure to the pesticide. Furthermore, this obesity-like phenotype was strongly correlated with plasma leptin levels. Coupled with this, some studies with human apoE-TR mice have also suggested that *APOE3* genotype contributes to the development of diet-induced obesity and related metabolic dysfunctions (Arbones-Mainar et al. 2008; Huebbe et al. 2015; Karagiannides et al. 2008). Indeed, apoE3 but not C57BL/6 mice developed hyperleptinemia following a 24-week exposure to a western-type diet (Karagiannides et al. 2008). Increased circulating leptin levels observed in CPF-exposed apoE3 mice might arguably increase the basal activity of JAK2/STAT3 signalling in neurons of the central nervous system. This process would

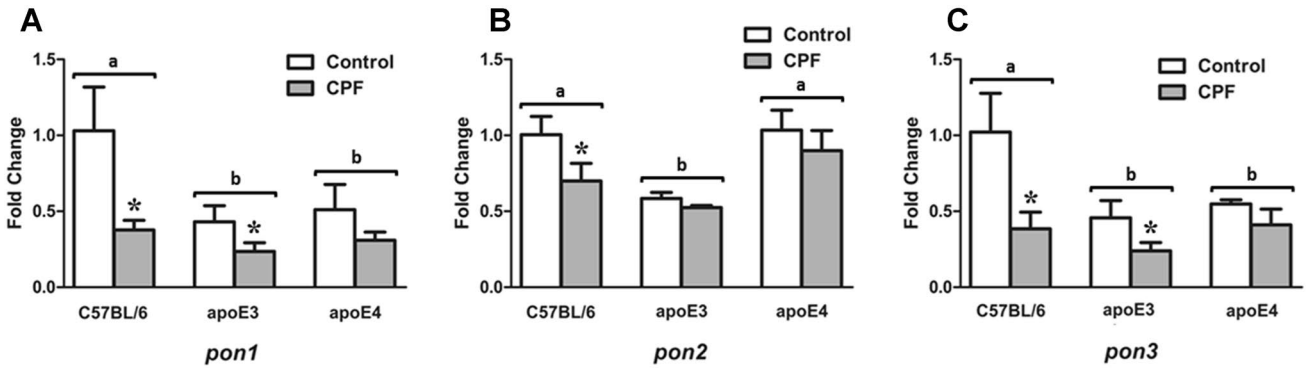

Fig. 4 We determined the relative hepatic expression of the mRNAs encoding **a** *pon1*, **b** *pon2*, and **c** *pon3* genes following an 8-week dietary exposure to CPF in apoE3, apoE4 and C57BL/6 adult male mice. Groups showing different letters (**a, b**) differ significantly from each other at $p < 0.05$. The symbol in the lower panel indicates effects of the treatment within each group at $p < 0.05$ (*)

in turn enhance the otherwise silent SOCS3 protein expression, thus ultimately further impairing LepRb sensitivity (Münzberg 2009).

Although increasing epidemiological and experimental evidence suggests that OPs cause insulin resistance, leading ultimately to T2D (Lasram et al. 2014), a great deal of uncertainty about their mechanism of action remains. Furthermore, only a few studies have explored the impairment of the insulin-signalling pathway after adulthood exposure to CPF in rodents (Acker and Nogueira 2012; Elsharkawy et al. 2013; Reygner et al. 2016), being most existing research focused on early-life stages (Lassiter and Brimijoin 2008; Slotkin et al. 2005). Moreover, some of these studies cited only reported changes at the hormonal level, and did not study the underlying mechanisms in depth. We recently showed that apoE3 adult male mice developed a sharper hyperinsulinemia than their C57BL/6 peers, and exhibited associated increases in plasma glucose following repeated exposure to 2 mg/kg body weight CPF (Peris-Sampedro et al. 2015b). The current results agree with our previous evidence (Peris-Sampedro et al. 2015b), demonstrating that dietary exposure to CPF leads to clear signs of insulin resistance in apoE3 adult male mice. Although the phosphorylated form of IRS-1 was not affected by the treatment, the expression of both p-AKT and GSK3β proteins was notably and solely reduced in the liver of CPF-fed apoE3 animals. The decrease we observed in p-GSK3β protein expression implies a down-regulation in the synthesis of glycogen due to an increase in the activity of GSK3β and consequently the inactivation of glycogen synthase enzyme. In agreement with the current results, some in vitro studies have confirmed the inhibitor role of CPF on p-AKT levels in different types of cells (de Oliveira et al. 2016; Lee et al. 2014; Schäfer et al. 2013). Several authors have also consistently reported a diminished content in hepatic glycogen of rats both acutely (Elsharkawy et al. 2013) and chronically exposed to CPF (Goel et al. 2006). These authors also described a significant inhibition of hepatic glucose uptake and other alterations in carbohydrate metabolism, such as significant increases in glucose-6-phosphatase and glycogen phosphorylase activities (Elsharkawy et al. 2013; Goel et al. 2006).

Unexpectedly, the phosphorylation ratio of IRS-1 remained unchanged in apoE3 mice after CPF exposure. However, we did notice that variations in such p-IRS-1 levels were positively and strongly correlated to those of the total protein. Accordingly, albeit not significantly, CPF-fed apoE3 mice seemed to express total IRS-1 protein to a lesser extent than the other two genotypes. Among the various mechanisms that control the insulin action, SOCS proteins are known to act as negative regulators by either inhibiting the tyrosine kinase activity of the IR, or targeting the IRS proteins for degradation (Rui et al. 2002; Ueki et al. 2004). Under inflammatory states or metabolic stress, many proinflammatory cytokines, including leptin and IL-6, upregulate SOCS proteins (Rui et al. 2002). Rui et al. (2002) reported that SOCS3 is likely to promote insulin resistance by targeting IRS-1 for ubiquitin-mediated degradation. The increased SOCS3 protein expression we observed in CPF-exposed apoE3 mice could, therefore, at least partially explain the slight changes in the total and consequently the p-IRS-1 protein expression. Further support for this idea has come from a number of authors, who have found increased SOCS3 protein expression in insulin target tissues of obese mice, which were correlated with reduced levels of IRS-1 and insulin resistance (Emanuelli et al. 2001; Kido et al. 2000). Likewise, inactivation of SOCS3 in LepRb-expressing cells protects mice from insulin resistance (Pedroso et al. 2014).

Oxidative stress reflects an imbalance between the production and elimination of reactive oxygen species (ROS) and it is known to contribute to both insulin resistance and T2D (Eriksson 2007). The most widely studied CPF toxic effects include the increase in ROS production in plasma

and organs, which leads to lipid peroxidation and increased protein carbonyl levels, ultimately prompting the inactivation of antioxidant defences in a concentration-dependent manner (Cacciatore et al. 2015; Gultekin et al. 2000; Mosbah et al. 2016). Various studies have demonstrated that a basal level of ROS induces the phosphorylation of AKT, thereby protecting cells from oxidative stress-mediated damage, whereas high levels of ROS downregulate the AKT-signalling pathway. Indeed, AKT has a kinase domain that is subject to oxidation events, ending with the inactivation of its inherent kinase activity by forming a disulfide bond between Cys-297 and Cys-311 (Murata et al. 2003). Interestingly, co-exposure of CPF with antioxidants, including glutathione, zinc, vitamins C and E, or compounds that stimulate the PI3K/AKT pathway, largely prevents the molecular alterations induced by the pesticide (de Oliveira et al. 2016; Elsharkawy et al. 2013; Goel et al. 2006; Gultekin et al. 2001; Narra et al. 2015; Olsvik et al. 2015). On the other hand, it has been reported that GSK3β phosphorylates the nuclear factor (erythroid-derived 2)-like 2 (Nrf2), thus inhibiting its translocation to the nucleus and the subsequent expression of antioxidant proteins. Likewise, the GSK3β activity blockade by PI3K/AKT enables the Nrf2 nuclear translocation and up-regulates detoxifying enzyme levels (Rojo et al. 2008). It is, therefore, tempting to speculate that the increased GSK3β activity we found in CPF-treated apoE3 mice might trigger an increase in ROS production via the down-regulation of the PI3K/AKT-signalling pathway and the subsequent blocking of Nfr2 nuclear translocation.

To date, there are still few references that support the obesogenic effect of CPF during adulthood (Ehrich et al. 2004; Meggs and Brewer 2007; Peris-Sampedro et al. 2015a, b). Meggs and Brewer (2007) pointed out that an increase in adipose tissue was the cause of the weight gain they observed in rats subjected to a subchronic exposure to low doses of CPF (Meggs and Brewer 2007). Accordingly, Howell et al. (2016) found that CPF significantly increased neutral lipid accumulation in a concentration-dependent manner in McA-RH7777 hepatocyte cells (Howell et al. 2016). Moreover, limited experimental studies with human apoE-TR mice have suggested an increased vulnerability of apoE3 mice to diet-induced obesity and related metabolic dysfunctions (Arbones-Mainar et al. 2008; Huebbe et al. 2015; Karagiannides et al. 2008). ApoE3 mice on a western-type diet (Arbones-Mainar et al. 2008; Karagiannides et al. 2008) or a control diet (Huebbe et al. 2015) were phenotypically more obese and exhibited increased fat depots than apoE4 mice. According to Huebbe et al. (2015), the reason for these differences is that apoE3 individuals are more prone to accumulating fat in adipose tissue, owing to their efficiency at harvesting dietary energy (Huebbe et al. 2015). Based on the above, it is reasonable to expect an additive effect with a combination of being carrier of the *ε3* allele and being exposed to CPF (Peris-Sampedro et al. 2015b). Sandhu et al. (2017) recently revealed that CPF accentuated the effect of retinoic acid (RA), a known cell differentiation agent, ultimately promoting the adipogenic differentiation of C3H10T½ cells (Sandhu et al. 2017). Furthermore, the authors found that both lipid differentiation and accumulation were dependent on GSK3β activation; a co-treatment with lithium chloride, a selective inhibitor of GSK3β activity, abolished these effects. A conceivable hypothesis is that the increased GSK3β activity here found exclusively in CPF-fed apoE3 mice might be not only diminishing glycogen synthesis in those individuals, but also favouring their lipid accumulation and subsequent weight gain.

The results of the present investigation provide novel and potential information about the effects of repeated adulthood exposures to CPF on the hepatic gene expression of *pon1, pon2*, and *pon3*. Interestingly, exposure to the pesticide reduced the expression of the three genes overall, but most notably in both apoE3 and C57BL/6 mice. In line with our results, Medina-Díaz et al. (2017) have recently shown that *pon1* mRNA in HepG2 cells decreased after 24, 48 and 72 h of CPF treatment (Medina-Díaz et al. 2017). Along the same lines, Acker and Nogueira (2012) reported a reduction in PON1 activity following a single acute dose of 50 mg/kg in rats. An exhaustive search of the scientific literature revealed that there is no single item of experimental evidence on the effects of CPF exposure on pon3 mRNA, and as such it remains an important subject for future research. As stated above, recent results from our group suggest that CPF-treated apoE3 are more vulnerable to developing obesity (Peris-Sampedro et al. 2015a, b). It has been recently shown that PON3 knock-out mice are more prone to gaining weight compared to their wild-type littermates when fed a high-fat diet (Shih et al. 2015). Based on all these data, we infer that the reduction in *pon3* expression particularly observed in apoE3 mice after CPF exposure may be contributing to the *APOE*-dependent obesogenic phenotype.

The potential toxicity of low-dose CPF has been sometimes under discussion, partly because of contradictory experimental and epidemiological outcomes. For example, while its potential in causing embryotoxicity and developmental disorders has been recurrently demonstrated in vitro (Estevan et al. 2013; Flaskos et al. 2011), the effects of the pesticide on human neurodevelopment remain controversial (Eaton et al. 2008). Therefore, humanized animal models, such as the apoE-TR mice, should be considered as a powerful tool to bridge the existing gap between experimental and epidemiological toxicology.

In summary, the current results, together with recent data (Peris-Sampedro et al. 2015a, b), show that apoE3 mice are the most vulnerable to the detrimental metabolic effects of CPF. These individuals manifested clear signs of insulin resistance in the liver following an 8-week dietary

exposure to 2 mg/kg body weight CPF, as revealed by the up-regulation of the JAK2/STAT3/SOCS pathway and the down-regulation of both AKT and GSK3β proteins. Taken together, these results point to an inhibition of the antioxidant mechanisms of the cell and a reduction in glycogen synthesis. These effects probably explain the T2D-like phenotype previously reported in apoE3 mice (Peris-Sampedro et al. 2015b). Furthermore, we provide the first evidence that dietary exposure to CPF generally decreases the expression of *pon1, pon2* and *pon3*, thereby paving the way for further lines of research on the metabolic-disruptor role of the pesticide. If the debate is to be moved forward, further studies are needed to determine whether the effects observed are attributable to the parental compound itself or to its metabolites (e.g., CPF-oxon). Finally, we have demonstrated that the effects of the pesticide on insulin- and leptin-signalling pathways are *APOE*-dependent, which highlights the importance of studying genetic risk factors in societies already burdened with an increased incidence of non-communicable chronic diseases.

Funding This research was supported by the Ministry of the Economy and Competitiveness (MINECO, Spain) (Grant Numbers PSI2010-21743-C02-01 and PSI2014-55785-C2-2-R) and the Commission for Universities and Research of the Ministry of Innovation, Universities and Enterprise of the *Generalitat de Catalunya* (Grant Number 2013 FI_B00170).

Compliance with ethical standards

Conflict of interest The authors declare that no conflict of interest has influenced the results presented in this investigation.

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