Diminished phosphorylation of CREB is a key event in the dysregulation of gluconeogenesis and glycogenolysis in PCB126 hepatotoxicity

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Abstract

The dioxin-like PCB126 elicits toxicity in various target organs. In rat liver an alteration in the transcript levels of several genes involved in glucose and fatty acid metabolism provides insights into the origin of its hepatotoxicity. To explore these, male Sprague-Dawley rats, fed AIN-93G diet were injected with PCB126 (1 or 5 µmol/kg) or corn oil and euthanized after 2 weeks. PCB126 significantly decreased serum glucose levels and the transcript levels of genes of many gluconeogenic and glycogenolytic enzymes under the transcriptional control of a nuclear transcription factor, cAMP response element-binding protein (CREB). As a novel finding, we show that PCB126 significantly decreases CREB phosphorylation, which is important for regulating both gluconeogenesis and fatty acid oxidation in the liver and explains CREB's integrative effects on both carbohydrate and lipid metabolism in PCB126 toxicity.

Graphical Abstract
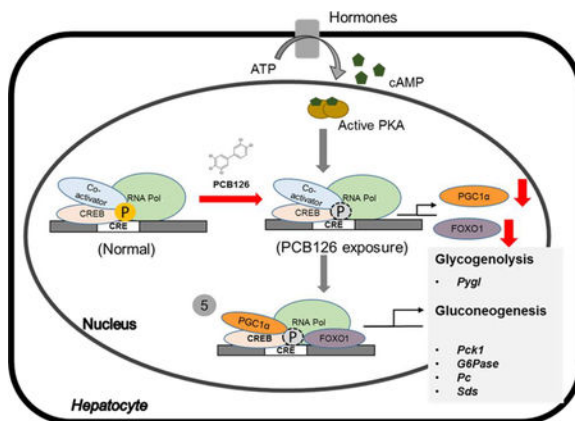
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Keywords

PCB126; dioxin; CREB; gluconeogenesis; glycogenolysis; metabolic disruptor; hepatotoxicity; fatty liver; glucose

Introduction:

Exposure and serum levels of a PCB (Polychlorinated biphenyl) congener, PCB126 and other dioxin-like compounds in human populations are positively correlated with altered blood glucose levels, risk of diabetes and non-alcoholic fatty liver disease (NAFLD).[1–3] PCB126 is resistant to biotransformation, accumulates in the liver and causes dioxin-like toxicity through activation of nuclear transcription factor aryl hydrocarbon receptor (AhR).[4] PCB126 binds to AhR and trans-activates the expression of genes and proteins that possess xenobiotic recognition elements (XREs) in their promoters.[5] The rapid transcription of enzymes involved in xenobiotic metabolism and the associated oxidative stress, does not completely explain the observed metabolic disruption and fatty liver in PCB126 exposure. [6, 7] We and several others have reported alterations of genes involved in gluconeogenesis and fatty acid oxidation.[8, 9] Despite the indication of effects of dioxin-like chemicals on intermediary metabolism and gluconeogenesis in early reports, very little is known about the mechanisms.[10–12] Hence, to study the genes that are involved in hepatic glucose production and consequent hypoglycemia, we have analyzed dose-dependent changes in the livers of rats acutely exposed to PCB126.

Materials and methods:

Animal studies:

All experiments were conducted with the approval from Institutional Animal Care and Use Committee of the University of Iowa. Male Sprague-Dawley (SD) rats weighing 75–100 g at an age of 4–5 weeks were fed a defined AIN-93G diet purchased from Harlan Laboratories (Indianapolis, Indiana) and acclimatized for three weeks. The animals were housed individually in wire hanging ages with free access to feed and water and in a controlled environment of 22°C in a 12 h light-dark cycle. Animals were randomly divided in 3 groups (6–7 rats per group) that received a single i.p. injection of vehicle (corn oil; 5 ml/kg body weight) or PCB126 at a dose 1 μmol/kg (326 μg/ kg body weight) or 5 μmol/kg (1.63 mg/ kg body weight) body weight. The PCB126 used in the injections was prepared by an improved Suzuki-coupling method as previously described.[13] The tocopherol stripped corn oil used to prepare PCB126 and vehicle injections was purchased from Acros Chemical Company (Pittsburgh, Pennsylvania). Two weeks after injection, all the animals were euthanized at the same time without fasting, using carbon-dioxide asphyxiation followed by cervical dislocation. Serum fractions separated from the whole blood obtained from the heart and the livers were harvested and flash frozen in liquid nitrogen and stored at – 80°C until further analysis.

Serum preparation and glucose measurements.

The collected blood was allowed to clot in the non-anticoagulant coated tubes and the serum fractions were separated by centrifugation at $1500 \times g$ for 10 min. The glucose levels in the serum were measured by using Glucose hexokinase (HK) assay reagent kit (G 3293) purchased from Sigma-Aldrich; glucose measurements were performed as described by the manufacturer. Briefly, the frozen serum was thawed on ice and diluted (1:10) with 1X PBS. 100 μl of each sample was added to clear bottom microtiter plate along with HK reagent and was incubated for 15 min on shaker. Absorbance of the samples was measure at 340 nm using a plate reader.

Gene expression analysis.

Total RNA of each rat liver sample or cell fraction was extracted using the RNeasy extraction kit from Qiagen Inc (Valencia, California). Briefly, 20–30 mg of liver tissue or cell lysate was homogenized and subjected to RNA extraction as described in the manufacturer's protocol. Absorbance of the isolated RNA was determined spectrophotometrically at 260 and 280 nm. RNA samples with purity ratios (A260/A280) between 1.8 and 2.0 were used for generating complementary DNA (cDNA) employing a high-capacity cDNA reverse transcription kit from Applied Biosystems Inc. (Foster City, California), as described. The real-time quantitative PCR analysis was performed at an optimized cDNA template concentration of 50 ng, using an SYBR Green Master Mix kit supplied by Applied Biosystems Inc. The primers used to measure the transcript levels of various genes are listed in Supplementary Table 1 and were synthesized by Integrated DNA Technologies Inc. (Coralville, Iowa). Each sample was analyzed in duplicate. The amplification reaction was carried out with an Eppendorf RealPlex2 Mastercycler (Hamburg, Germany) using a program that started at 95 for 10 min followed by 40 cycles of 2 step PCR cycle at 95° C for 15 s and 60° C for 1 min. Subsequently, a melting curve analysis was also performed. The transcript levels of all the quantified genes were normalized to the transcript levels of the reference gene hypoxanthine-guanine phosphoribosyl transferase *(Hprt1)*. The expression level of each gene in a given sample was normalized to the mean of the biological control group of each study (oil vehicle 5 ml/kg). The final transcript levels were quantified relative to the normalized transcript levels of the control group, using the Pfaffl method.

Protein isolation and semi-quantitative analysis

Liver snippets (~ 50 mg) were homogenized in lysis buffer (500 μl) containing phosphatase and protease inhibitors. The homogenates were sonicated for 2 min (in rounds of 10 s sonication/20 sec rest cycle) and incubated on ice for 30 min. Supernatants were collected after centrifugation at 10000 rpm for 20 min at 4°C and stored at −20°C. Total protein was measured quantified by the Bradford assay as described by manufacturer (Biorad, CA). Proteins were resolved by denaturing 10% SDS-polyacrylamide gel electrophoresis and transferred by wet blotting onto PVDF membranes (Millipore, MA). The membranes was blocked with 5% non-fat milk or 5% BSA in 25 mm Tris-HCl (pH 7.4), 150 mm NaCl and 0.1% Tween-20 (TBST) at room temperature for 1 h, and incubated with primary antibody (overnight) and with secondary antibody (1 h) diluted in non-fat milk or BSA, as

recommended by the supplier. The secondary antibodies conjugated with HRP were used to visualize the blots with a chemiluminescence Supersignal West Pico and West Femto kit (Pierce Chemical Co.) and observed on a LI-COR Odyssey chemiluminescent imager. For stripping of the antibodies bound to the blot, the blots were incubated with Restore™ western blot stripping buffer (Thermo scientific, MA) for 30 min at 60°C with agitation and then washed liberally for five times with TBST. The membranes were probed with the primary antibodies to the following: Rabbit monoclonal antibody against CREB phosphorylated at S133 (9198; Cell signaling), Rabbit monoclonal antibody against CREB (9197; Cell signaling), goat polyclonal antibody against PEPCK-C (gift of Dr. Daryl J. Granner), rabbit polyclonal antibody against the β-actin (ab8227; abcam). The specificity of pCREB and CREB antibodies was validated by probing for the protein levels of CREB and pCREB in unstimulated (-ve control) and stimulated (+ve control) neuronal cell extracts from SK-N-MC cells, untreated or treated with Forskolin (30 μM), IBMX (0.5 mM) for 30 min to enhance CREB phosphorylation. The band intensities of all the probed proteins were analyzed with ImageJ analysis software.

Statistics

The differences across the control and various treatment groups were analyzed using a one-way ANOVA followed by Tukey's *post hoc* test. The outliers in the sample distribution were determined using Grubs test and eliminated. Only results with significant differences ($P < .05$) were reported. All the error bars represent standard deviation (SD) of the mean. All the statistical analyses were performed using GraphPad Prism software (GraphPad Software, Inc, CA).

Results and Discussion:

Serum glucose levels in rats treated with PCB126 were significantly decreased (figure 1A) at the higher (5 μmol/kg) dose, but no changes were seen at lower dose (1 μmol/kg). Liver plays an important role in maintaining glucose homeostasis by producing glucose especially in fasted states. This is achieved through glycogenolysis and gluconeogenesis.[14] The rate limiting enzyme necessary for gluconeogenesis is phosphoenol-pyruvate carboxykinase (PEPCK-C/PCK1).[15–18] PEPCK-C catalyzes the conversion of oxaloacetate (OAA), a tricarboxylic acid (TCA) cycle intermediate into phosphoenolpyruvate (PEP) which is subsequently converted into glucose and exported out of the liver. The rate of gluconeogenesis in the liver is regulated by modulating the expression ofPEPCK-C through transcription.[19] Exposure to PCB126 results in significant dose-dependent decrease in the protein levels of PEPCK-C (figure 1B) by at least 80% (figure 1C), compared to the control animals (n=4). The effects of PCB126 on decreasing the transcript levels of PEPCK-C were previously reported to occur as early as 9 hrs.[20] The protein levels of PEPCK-C and transcript levels of several other enzymes such as glucose-6-phosphatase (*G6pase*) that play an important role in hepatic glucose production during gluconeogenesis were also down-regulated in a dose-dependent fashion (figure 2B).[21]Despite the decreasing trend in these enzymes, the lack of reduction in serum glucose levels at lower dose of PCB126 may be explained by the compensatory replenishment of glucose that results from feeding (feed was available at all times).

During shorter durations of fasting, the demand for glucose is fulfilled by catabolizing the stored glycogen and converting it into glucose through glycogenolysis. Glycogen phosphorylase (*Pygl*) that catalyzes the glycogenolysis by releasing glucose-1-phosphate from the polymeric glycogen stored in the liver is also decreased during PCB126 exposure (figure 2A). An interesting finding from the current and previous studies that corroborates the decrease of *Pygl*, is the presence of glycogen in the livers of PCB126-treated animals despite the physiological demand for glucose during the observed hypoglycemia.[20, 22]

In prolonged starvation depleted glycogen or inefficient glycogen metabolism, the liver shifts into gluconeogenesis to replenish glucose. While cataplerotic conversion of the TCA cycle intermediate OAA into PEP by PEPCK supports gluconeogenesis, the regeneration of OAA through anaplerosis of various substrates such as pyruvate and certain gluconeogenic amino acids, such as serine, is equally important to meet the demand for glucose production.[23] Anaplerotic conversion of pyruvate during gluconeogenesis is catalyzed by pyruvate carboxylase (*Pc*), to generate OAA.[24] More complex substrates such as serine, involve a sequential two-step conversion of free amino acid serine into pyruvate by an initial action of the enzyme serine dehydratase (*Sds*).[25] Along with PEPCK-C and G-6-Pase, that have well-known roles in hepatic glucose production, exposure to PCB126 also significantly reduces the transcript levels of the enzymes *Pc* (figure 2C) and *Sds* (figure 2D), involved in anaplerotic conversion of pyruvate and serine into OAA during gluconeogenesis. These observations show that PCB126-induced decrease in hepatic glucose production occurs due to its effects on PEPCK-C and multiple enzymes in the gluconeogenic pathway.

Glucose production in the liver is a tightly regulated by selective transcription of target enzymes necessary to metabolize the gluconeogenic substrates. The selectivity is attained by activation of specific nuclear receptors (NR) that interact with specific transcriptional co-activator proteins, necessary to recruit the RNA polymerase II. The transcription of distinct target genes during gluconeogenic signal transduction is thus achieved through inclusion of transcription coactivators such as peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α).[26, 27] Previous studies have illustrated that *PGC-1α*, a transcriptional coactivator is highly induced during fasting and regulates gluconeogenesis.[28] The expression of PGC-1α is controlled by transcriptional activation of cAMP response element binding protein1 (CREB1), in response to various hormonal stimuli that increase the cAMP levels in the cell.[29, 30] The increased cAMP levels activate the phosphorylation of CREB1 (pCREB), which renders the assembly of PGC1α and the transcription initiation complex necessary for the specific transcription of gluconeogenic genes during fasting.[29] We found that PCB126 treated animals, have significant reduction in the transcript levels of *Pgc1α*, necessary for gluconeogenesis (figure 3A). In addition to PGC1α, another transcriptional coactivator that is important for hepatic gluconeogenesis is Forkhead box protein O1 (FOXO1). Liver specific deletion of *Foxo1* results in reduced blood glucose levels, decreased hepatic glucose production and reduced expression of gluconeogenic genes including *Pepck-c, G6Pase* and even *Pgc1α*.[29, 31, 32] Although the mechanisms are unclear, several studies have demonstrated that the conjunction of FOXO1 and PGC1α is critical in mediating the transcription of gluconeogenic genes during starvation.[31–33] The livers of PCB126-treated rats with decreased expression of functional gluconeogenic enzymes in this

study, also show a dose-dependent decrease in the transcript levels of both the transcriptional co-activators, *Foxo1* and *Pgc1α*. gure 2B).

Despite, the necessity of several transcriptional coactivators such as *Foxo1* and *Pgc1α* for the specificity and signal discrimination during cell signaling and transcriptional targeting of gluconeogenic genes, an upstream activation of CREB is necessary to activate hepatic glucose production.[34] Activation of CREB is regulated through phosphorylation of a critical serine residue (S133) by protein kinase A (PKA) in the presence of cAMP.[35] pCREB, localized on the promoters of genes with the cAMP response element (CRE), enables the recruitment of the transcriptional coactivators PGC1α and FOXO1. We have probed for the activity of CREB1 by using a specific antibody against pCREB1 (S133) and found that PCB126 significantly decreases the phosphorylation of CREB1 (figure 4). The effects of PCB126 on phosphorylation were dose-dependent (figure 4A and 4B) and did not alter the total protein levels (Figure 4A) nor the transcript levels of CREB1 (supplementary figure 2B). The phosphorylation of CREB1 is the hallmark of cAMP-stimulated signaling necessary for the activation of distinct programs of gene expression across various cell types under different hormonal stimuli.[35, 36] CREB acts as a metabolic sensor that precisely controls the expression of several genes in the liver, in response to feeding and fasting signals.[26] Phosphorylation thus facilitates a reversible mechanism to regulate genes involved in gluconeogenesis and fatty acid oxidation, in order to provide metabolic homoeostasis of carbohydrate, lipid and amino-acid nutrients. Several studies performed by inactivation of genes encoding *Creb* or its coactivator *Pgc1α* and *Foxo1* have demonstrated dyshomeostasis in liver metabolism through impaired glucose production and non-alcoholic fatty liver.[32, 33, 37] While pCREB directly interacts with PGC1α to transcribe gluconeogenic genes, it also plays an indirect role on lipid metabolism by prolonging the transcription of *Pgc1α* during extended fasting or starvation. The translated PGC1α hetero-dimerizes with another nuclear receptor PPARα, to transcribe genes necessary for β-oxidation of fatty acids during energy deprivation. PPARα is regarded as the master regulator of fatty acid oxidation.[38,39]

The transcriptional changes in genes that occur after AhR binding to XREs in their promoters may explain certain mechanisms of PCB126 toxicity, but the alterations in the expression of genes, that lack XREs or functional AhR binding, adds complexity to the understanding of PCB126-induced liver pathology. AhR-activity has been previously reported to disrupt other signaling pathways by interfering with the transcriptional programming of NRs such as PPAR, Nrf2 and estrogen receptor; by altering their expression levels or increase the competition across their coactivators through "squelching".[20, 40–44] In light of these novel findings of PCB126-induced effects on CREB phosphorylation, further research is warranted on understanding the cross-talk between AhR activation and CREB signaling.

In conclusion, these studies suggest that exposure to PCB126 causes a decrease in serum glucose levels by impeding the expression of enzymes necessary for gluconeogenesis and glycogenolysis. The decrease in transcription of these enzymes, critical in carbohydrate and lipid metabolism in PCB126 toxicity, is regulated at the level of CREB phosphorylation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

ABBREVIATIONS

CREB   cAMP response element-binding protein

FOXO1   Forkhead box protein O1

PGC1α   Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (Pgc1α)

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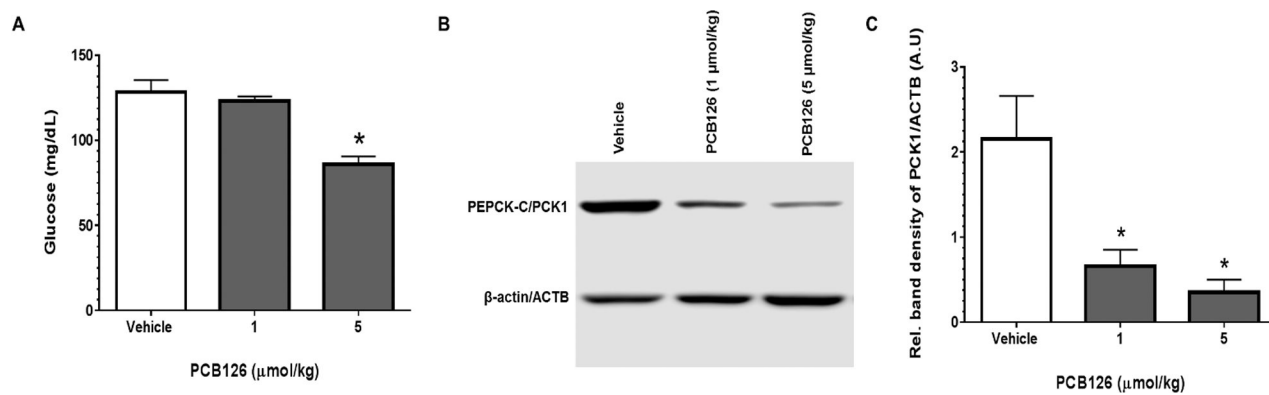
Figure 1. PCB126 decreases serum glucose and the protein levels of PEPCK-C.
Exposure to PCB126 caused a (A) decrease in serum glucose levels and a (B) dose-dependent reduction in protein levels of PCK1/PEPCK-C. The dose-dependent (C) decrease in PEPCK-C/PCK1 levels was quantified by densitometry using ImageJ analysis (* represents $P < 0.05$; one-way ANOVA).
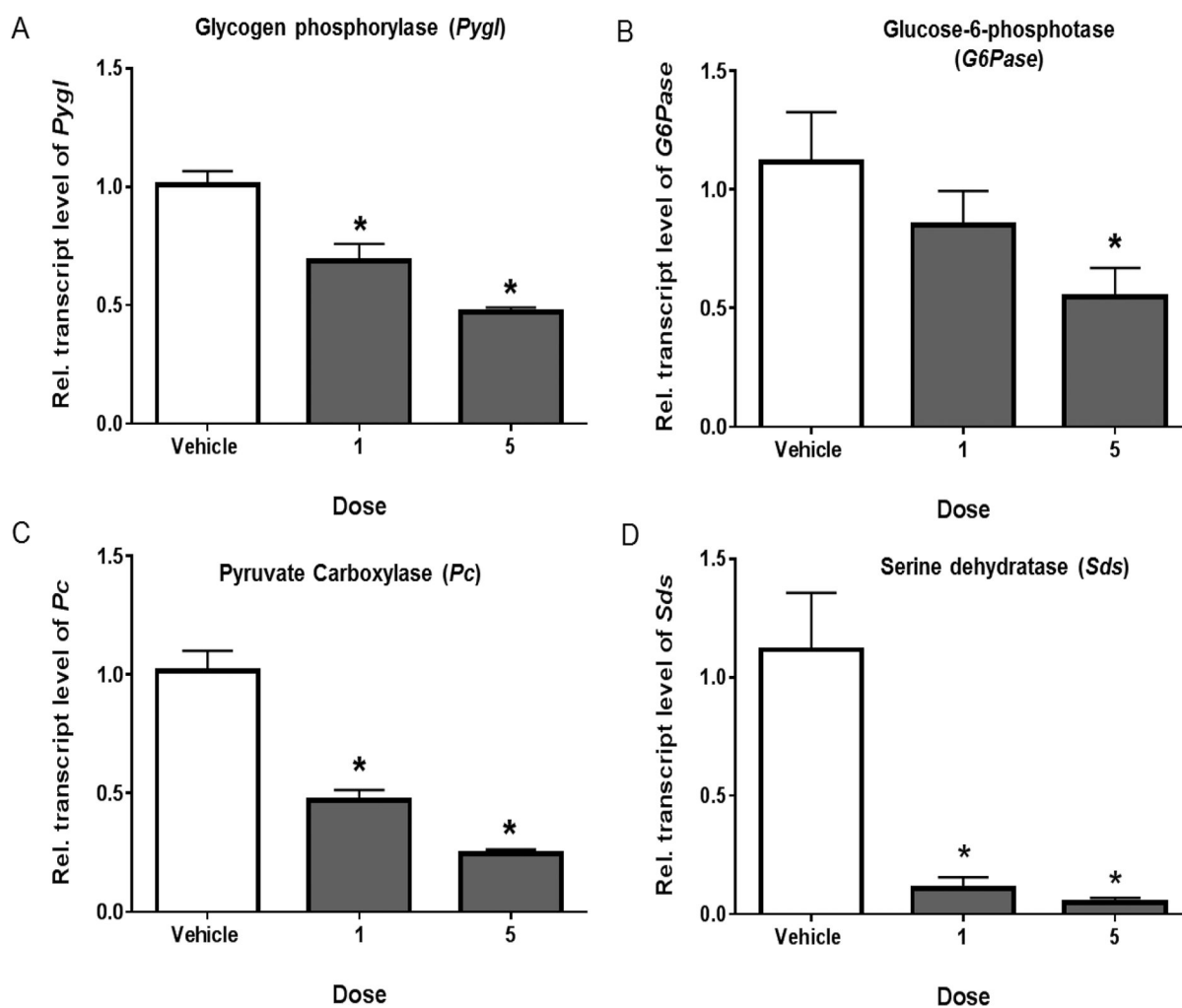
Figure 2. PCB126 downregulates the transcript levels of genes involved in glycogenolysis and gluconeogenesis.
PCB126 dose-dependently down regulates the transcription of (A) Glycogen phosphorylase (*Pygl*) involved in glycogenolysis and (B) Glucose-6-phosphotase (*G6Pase*), (C) Pyruvate carboxylase (*Pc*), (D) Serine dehydratase (*Sds*) involved in gluconeogenesis (* represents P < 0.05; one-way ANOVA).
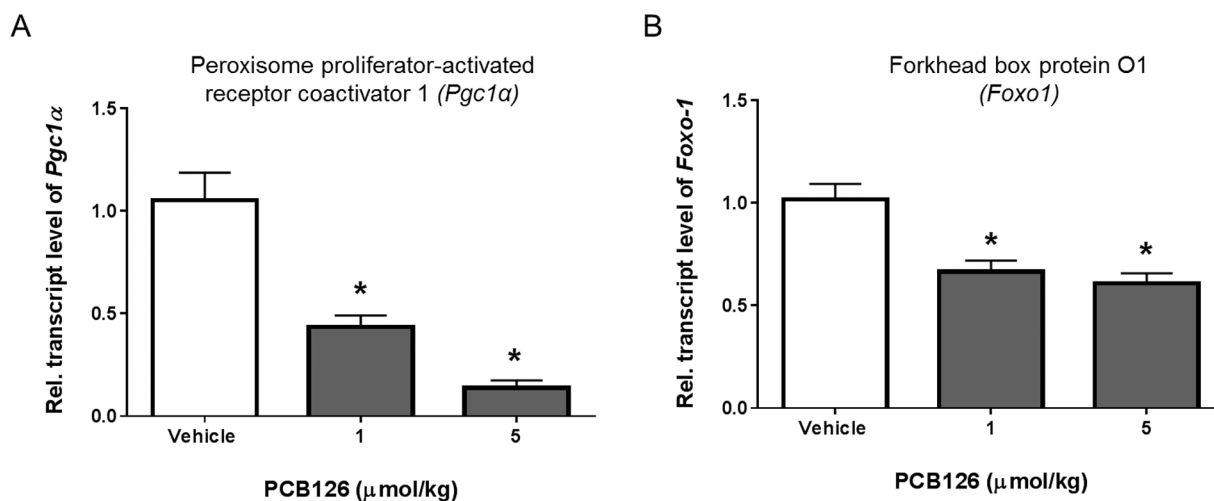
A

Peroxisome proliferator-activated receptor coactivator 1 *(Pgc1α)*

B

Forkhead box protein O1 *(Foxo1)*

Figure 3. PCB126 downregulates the transcript levels necessary transcriptional coactivators during gluconeogenesis.

PCB126 decreases the transcript levels of (A) proliferator-activated receptor gamma coactivator 1-alpha (*Pgc1α*) and (B) forkhead box protein O1 (*Foxo1*), necessary for transcription of functional genes involved in gluconeogenesis (* represents P < 0.05; one-way ANOVA).
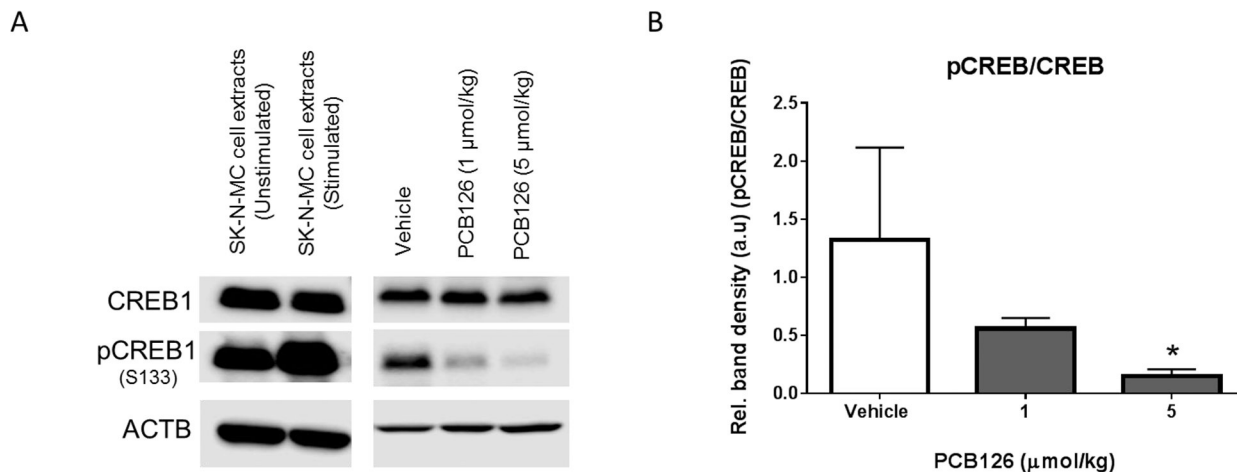
Figure 4. PCB126 dose-dependently inhibits the activation of hepatic CREB1.
Exposure to PCB126 results in reduced CREB phosphorylation (pCREB) on serine (S133) *(A; center panel)*, but does not change the total protein levels of CREB *(A; top panel)*. ACTB *(A; bottom panel)* represents the β-actin (control). The (B) ratio of pCREB/CREB were normalized to ACTB and found to be significantly decreased. Neuronal SK-N-MC cells stimulated with forskolin were used as a control for *(A; left panel)* specific recognition of pCREB during CREB activation.