PCB153 reduces apoptosis in primary cultures of murine pituitary cells through the activation of NF-$\varkappa$B mediated by PI3K/Akt.

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Abstract

Polychlorinated biphenyls (PCBs) are persistent pollutants involved in human tumorigenesis. PCB153 is a ubiquitous non-dioxin-like PCB with proliferative and anti-apoptotic effects. To explore the impact of PCB153 in the survival of pituitary cells, we exposed murine pituitary primary cells to PCB153 10 μM for 24h. Apoptosis was assessed by RT-qPCR, Western-blot, immunoprecipitation, caspase activity, and immunofluorescence. We found that PCB153 decreased pituitary apoptosis through both the extrinsic and intrinsic pathways. PCB153 reduced the level of the pro-apoptotic protein p38-MAPK. Otherwise, PCB153 activated PI3K/Akt and Erk1/2 pathways and enhanced the expression and nuclear translocation of NF-κB. Cotreatments with specific inhibitors revealed that only PI3K/Akt changed the caspase-3 expression and NF-κB activation induced by PCB153. Also, PCB153 decreased the expression of the pro-apoptotic and pro-senescent cyclins p53 and p21. In summary, exposure to PCB153 leads to a downregulation of apoptosis in the pituitary driven by a PI3K/Akt-mediated activation of NF-κB.

Keywords

PCB153, apoptosis, pituitary, PI3K/Akt, NF-κB, p21/p53

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Ethical Committee approval

The local Committee for the Animal Experimentation approved the protocol of the study before its start (protocol n°: 11033/2017, University of Pisa).

Declaration of interest

Declarations of interest: none.

Highlights

- PCB153 is a non-dioxin-like PCB frequently found in biological samples.
- PCB153 has been associated with proliferative and anti-apoptotic effects.
- PCB153 reduced pituitary apoptosis acting by the extrinsic and intrinsic pathways.
- Akt, Erk, p38, and p21/p53 contribute to the pituitary effects of PCB153.
- PCB153 induced an Akt-mediated activation of NF-$\varkappa$B in the pituitary.

Abbreviations

- PCB: Polychlorinated biphenyl
- W: Wortmannin, a PI3K/Akt inhibitor
- PD: PD98059, an Erk inhibitor
- SB: SB203580, a p38-MAPK inhibitor

1. Introduction

Polychlorinated biphenyls (PCBs) are a class of persistent organic pollutants extensively used in the 20th century as dielectric fluids and coolants in electrical apparatuses, and as additives to hydraulic lubricants, carbonless copy paper, and paints (International Agency for Research on Cancer, 2015; Quinete et al., 2014).

Recognition of the detrimental effects of PCBs on health led to the progressive limitation of these substances and ultimately their worldwide ban in 2001. Nevertheless, PCBs remain a threat for living beings due to their chemical stability and the improper disposal of residual sources. This explains the persistence of measurable levels of PCBs in soil, water, and air samples from all over the world (Arinaitwe et al., 2018; Chakraborty et al., 2018; Hens and Hens, 2017; Kolarik et al., 2017; Nøst et al., 2019).

Humans can be exposed to PCBs not only in the workplace or the environment but also, indirectly, by the ingestion of contaminated foods. In fact, PCBs frequently contaminate biological tissues due to their great lipophilicity and tendency to bio-accumulate in the food chain through animal fats (Hens and Hens, 2017; International Agency for Research on Cancer, 2015; Quinete et al., 2014). It is therefore not surprising that traces of PCBs are still even now detected in human tissues and fluids (including milk) (Chen et al., 2017; Lauritzen et al., 2018; Müller et al., 2017; Quinete et al., 2014; van den Berg et al., 2017; Wielsøe et al., 2017).

The biological effects of PCBs are complex and vary according to the structure of the individual congener (i.e., dioxin- and non-dioxin-like PCBs), the dose, and duration of exposure. The response to PCBs also depends on the biological characteristics of the exposed organism (e.g. species, sex, age) or tissue. In humans, PCBs influence the immune, reproductive, nervous, and endocrine systems (International Agency for Research on Cancer, 2015; Quinete et al., 2014). They are also involved in the induction and progression of some cancers and are thus classified as humans carcinogens by the International Agency for Research on Cancer (IARC) (International Agency for Research on Cancer, 2015; Quinete et al., 2014; Zani et al., 2017).

The pituitary is a classic target for PCB toxicity (Brandt, 1975). Most studies have focused on the disruption of the endocrine function, showing that specific PCBs can affect the synthesis and release of pituitary hormones by the interaction with aryl hydrocarbon, estrogen, and thyroid hormone receptor signalling (Bansal and Zoeller, 2008; Bestervelt et al., 1998; Cocchi et al., 2009; Desaulniers et al., 1999; Gauger et al., 2007; Khan and Hansen, 2003; Kitamura et al., 2005; Otake et al., 2007). Together with the modulation of endocrine functions, PCBs have also been associated with effects on the death of pituitary cells, with discrepant results in relation to the specific congener and the type of treated cells (Fortunati et al., 2017; Ghisari and Bonefeld-Jorgensen, 2005; Johansson et al., 2006). In recent years, we have tested the impact of various PCBs on the cell fate of primary cell cultures from the murine pituitary. Our results suggest that the dioxin-like PBCs 77 and 126 do not affect apoptosis. On the other hand, apoptosis was increased or reduced by the non-dioxin-like PCBs 180 and 153 in the pituitary, respectively (Raggi et al., 2016).

PCB153 is currently considered as one of the most persistent PCBs due to its widespread use in the past and its great stability and propensity to bioaccumulate (Center for Disease Control and Prevention, 2018; Kraft et al., 2017; Pavuk et al., 2014). This has led to increasing focus on the biological effects of this congener, highlighting the anti-apoptotic and proliferative effects of PCB153 as indicated by in vivo (Liu et al., 2014; Tharappel et al., 2002) and in vitro studies (Abella et al., 2015; Ferrante et al., 2011; Ptak et al., 2011; Sánchez-Alonso et al., 2003).

The aim of the study was to examine the molecular mechanisms by which PCB153 exerts its anti-apoptotic effects in the murine pituitary. Our results indicated that PCB153 reduce pituitary apoptosis acting by both the death receptors and the mitochondrial pathways. We also found that the PCB153 anti-apoptotic action was induced by the activation of the PI3K/Akt pathway and by an Akt-mediated activation of NF-$\varkappa$B.

2. Materials and methods.

2.1. Animals

All the procedures were conducted in accordance with EC Directive 86/609/EEC for animal experimentation. The local Committee for Animal Experimentation approved the study protocol prior to its start (protocol n°: 11033/2017, University of Pisa).

As donors of pituitary tissues, we used male C57BL/6J X CBA mice aged from 8-12 weeks. The animals were housed in standard cages with free access to water and standard pellet chows. The animal room had controlled conditions with a 12-hour light/dark cycle, and stable humidity and temperature (45-55% and 20°C, respectively).

2.2. Chemicals, media, and buffers.

2,2',4,4',5,5'-Hexachlorobiphenyl (PCB153) was obtained from VWR International (Radnor, PA, USA); Cis-diamine platinum (II) dichloride (Cisplatin), Wortmannin (W), PD98059 (PD), SB203580 (SB), Nutlin-3 and all other chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise specified. We dissolved all listed chemicals in 0.1% DMSO, which was used as a vehicle.

For the pituitary cell culture, we used Neurobasal-A medium supplemented with 50X B-27 (Life Technologies, Carlsbad, CA, USA), 0.5 mM L-glutamine, 10% heat-inactivated fetal bovine serum, 2% penicillin-streptomycin and 1% amphotericin B (complete medium). A serum-free medium was made up in the same way as the complete one but without the fetal bovine serum. The lysis buffer used for the cell extracts contained 150 mM NaCl, 10 mM Tris-HCl (pH 7.4), 1 mM EGTA, 1 mM EDTA, 1% Triton X-100, and a 100X protease inhibitor cocktail (Complete™ Protease Inhibitor Cocktail, Roche Applied Science, Germany).

2.3. Antibodies

The complete list of all the antibodies used in the study is provided in Supplementary Materials (Supplementary Materials&Metods, section S1.1).

2.4. Primary cell cultures of the murine pituitaries

After sacrificing the mice, the anterior pituitaries were dissected, washed twice in HBSS, minced to 2-3 mm slivers, and dispersed to individual cells by incubating tissues under gentle agitation with 0.25% Trypsin type II-S, 0,5% Collagenase, and DNAase 1 μM in Neurobasal-A complete medium for 45 minutes at 37°C. Freshly dissociated cells were centrifuged (1000 RPM, at RT for 5 min), and resuspended in 1 ml of the complete medium for the cell count (Countess automated counter, Invitrogen, Carlsbad, CA, USA) and appropriate seeding.

For RT-qPCR, Western Blot and immunoprecipitation analysis, the pituitary cells were seeded in 35 mm cell culture dishes coated with poly-L-Lysine at a density of $5x10^5$ cells/ml. For the caspase activity assays, we seeded cells in 96-well tissue culture plates coated with poly-L-lysine at a density of $5x10^3$ cells/well in 100 μl of the complete medium. For immunofluorescence, cells were seeded in 8-chamber cell culture slides coated with poly-L-Lysine (BD Biosciences, San Jose, CA, USA), at a density of $2x10^5$ cells/slide in a 250 μl volume of complete culture medium.

2.5. Experimental protocols

After seeding, cells were incubated at 37°C in a humidified atmosphere (95% air, 5% CO2) for three days. The complete medium was then replaced with the serum-free medium and cells were incubated for further 24 hours with the addition of PCB153 (10 μM) or the vehicle alone used as a control. The treatment dose of the PCB153 was derived from preliminary studies showed in the supplementary material of the manuscript (Supplementary Figure S1) and was demonstrated to be non-cytotoxic for pituitary cells (Raggi et al., 2016). For the protein

expression and activity assays of caspases, we also considered a third group (Cisplatin, 32 μM), which was used as a positive control of apoptosis, in accordance with the literature (Raggi et al., 2016). To assess the role of different signalling pathways on the PCB153-dependent modulation of apoptosis and activation of NF-ϰB, we exposed cells to PCB153 alone or with specific inhibitors of the explored kinases. Wortmannin (W, 15 μM) was used as an inhibitor of the PI3K/Akt pathway, whereas PD98059 (PD, 25 μM) and SB203580 (SB, 10 μM) were used to inhibit Erk/MAPK and p38/MAPK pathways, respectively. The effects of PCB153 on p21/p53 pathway were assessed pre-treating or not the cells with PCB153 (10 μM) for 24 h and then with Nutlin-3 (10 μM) for 48 h (Chesnokova et al., 2013).

2.6. RNA extraction and RT-qPCR analysis

Total RNA was extracted from primary pituitary cells with TRIzol® reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol. The extracted RNA quality was assessed by the RNA 6000 Nano LabChip assay and an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Total RNA (1 μg) was transcribed into cDNA using Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA) and pd(N)6 random hexamer (GE Healthcare, Milan, Italy) in a final volume of 20 μl, following the manufacturer's protocol. The resulting cDNA was used to assess the expression of caspase-3, -8 and -9 mRNA in cells treated with PCB153 or the vehicle. qPCR was performed using SSO Advanced Universal SYBR® Green SuperMix (Biorad, Hercules, CA, USA), with the following cycling conditions: 95°C for 5 sec (denaturation) and 58°C for 30 sec (annealing/extension) repeated 40 times. GAPDH was chosen as a reference gene and data were analysed through the $2^{-\Delta\Delta Ct}$ method. The primers used are listed in Supplementary Material (Supplementary Materials and Methods, section S1.2).

2.7. Cell extracts, Western blot, and immunoprecipitation assays

After the treatments, primary cell cultures were washed twice with ice-cold PBS, collected by trypsinisation, pelleted and incubated on ice for 30 min with 200 µL of the lysis buffer. We then centrifuged homogenates, removed the supernatant, and measured the protein concentrations using the Bradford method (Bio-Rad Laboratories, Hercules, CA, USA). Protein extracts were used immediately or stored at -80°C for further analysis.

For the Western Blot, equal amounts of total pituitary cell extract (25 µg/lane) were separated on a 12% SDS-PAGE, transferred onto a nitrocellulose membrane, and stained with Ponceau red to verify the amount of protein in each lane. Residual protein-binding sites on the membrane were blocked in a 1:1 TBS-TTBS solution containing 5% (w/v) of non-fat dry milk for 2h at RT and incubated overnight at 4°C with the specific primary antibody (1:200-1:500). The membranes were then rinsed three times for 15 min with TTBS, and incubated with the appropriate HRP-conjugated secondary antibody (1:1000) for 1h at RT.

Immune-reactive protein was detected using an enhanced chemiluminescence detection system (Bio-Rad Laboratories, Hercules, CA, USA). Membranes were then incubated at 70°C for 10 min in a stripping buffer (5 mM Tris–HCl (pH 6.8), 2% SDS, 0.8% β-mercaptoethanol) and re-tested for β-actin. Films were scanned and bands intensity was evaluated using ImageJ v1.49 software (http://rsb.info.nih.gov/ij). Each sample value was normalised for loading errors (dividing the intensity of the band of interest by the intensity of β-actin, or Ponceau S staining). Data were expressed as arbitrary units.

For the immunoprecipitation assay, FADD antibody (5 µg) was incubated overnight at 4°C with 50 µg of total tissue extracts. We then incubated the mixture with 50 µl of a 1:1 suspension of n-protein A-Sepharose beads (GE Healthcare Bio-Sciences, Uppsala, Sweden) for 1h at 4°C with gentle rotation. The beads were pelleted and washed three times with the lysis buffer to remove non-specifically bound proteins. Finally, the immunocomplexes were separated by

boiling the mixture in an SDS-PAGE sample buffer and examined by Western blot, using caspase 8 (H-134) rabbit polyclonal IgG as the primary antibody.

2.8. Caspase activity assay

The caspase activity was assessed by fluorometric and luminometric assays using the NucView 488 Caspase-3 kit (Biotium; Hayward, CA, USA) and Caspase 8 or 9 Glo kit (Promega; Milan, Italy), respectively (Dickerson et al., 2011; Hsu et al., 2007). At the end of the treatment period, cells were incubated with a fluorogenic or luminogenic substrate for 30 minutes at room temperature, according to the manufacturer's instructions. Fluorescent and luminescent measurements were performed using a FLUOstar Omega microplate reader (BMG Labtech, Ortenberg, Germany). Data were expressed as arbitrary units attributing the value of 1 to the caspase activity of cells treated with the vehicle.

2.9. Immunofluorescence Microscopy, Confocal Imaging and image analysis

For the NF-κB immunofluorescence assay, cells were fixed in 4% paraformaldehyde for 20 min at RT, washed with PBS, blocked with BSA 1% in PBS and then incubated overnight with 1:100 NF-κB p65 (RelA) (F-6) mouse monoclonal primary antibody, followed by 1:500 goat anti-mouse Alexa Fluor 488 secondary dye antibody for 1h at RT. 4,6-diamidino2- phenylindole (DAPI) (ThermoFisher Scientific, Waltham, MA, USA) was used as a nuclear marker. Samples were directly observed by fluorescence microscopy using a x40 objective (Nikon Eclipse 80i; Nikon Corp., Japan), and images were captured using ACT-2U Nikon software (Nikon, Nikon Corp., Japan). The nuclear translocation of NF-κB p65 (RelA) was analysed using a quantitative method based on ImageJ software, as previously described (Noursadeghi et al., 2008). Briefly, binary masks of the nuclear and cytoplasmic region of interest (ROI) were created by the application of median filters and automatic thresholding on DAPI, and NF-κB p65 (RelA) previously acquired fields. These masks were subtracted from the original NF-κB p65 (RelA)

stained field using image calculators, obtaining cytoplasmic and nuclear NF-κB p65 (RelA) signals, respectively. Data were expressed as the nuclear/cytoplasmic NF-κB p65 (RelA) fluorescence intensity ratio.

For Cytochrome-C translocation experiments, before paraformaldehyde fixation cells were treated with MitoTracker® Deep Red FM (Invitrogen) 250nM for 30 minutes. After fixation, cells were permeabilised with PBST for 10 minutes. Blocking was performed with 1% BSA in PBST and 10% goat serum for 1h at RT, then mouse monoclonal Cytochrome-c primary antibody (A-88) 1:100 was incubated overnight at 4°C. Goat anti-mouse Alexa-Fluor 488 () was incubated 1:500 for 1h at RT. Fluorescence-based co-localisation experiments were performed using a Zeiss LSM 880 AiryScan confocal microscope equipped with a 63X 1.4 N.A. oil immersion objective and GaAsP detectors. Alexa 488 was excited at 488 nm and emission collected in the 500-600 nm range. MitoTracker Deep Red was excited at 640 nm and emission collected in 645-700 nm range. Each image consists of 1024 × 1024 pixels, with a pixel size of 50 nm.

Manders' co-localisation coefficients (M1 and M2) were calculated between Alexa 488 (green channel) emission and MitoTracker Deep Red (red channel) emission for each stack of images acquired, using the dedicated JaCoP plugin for ImageJ. The M1 coefficient was selected to quantify the overlap between the green and red signals (Manders et al., 1993).

2.10. Statistical analysis

Results were expressed as mean + SD of at least three independent experiments performed in duplicate. We used Prism v. 5.0 and v. 7.0 (GraphPad Software, San Diego, CA, USA) to perform data analysis and to represent the results graphically. The Shapiro-Wilk and Levene tests were used to evaluate the normality of continuous data and the homogeneity of the variance. A two-tailed unpaired Student's t-test was used for group comparisons between vehicle and PCB153 treatments in caspase activity, RT-qPCR, and Western Blot assays. In the

NF-κB nuclear translocation experiment, a two-tailed unpaired Student's t-test with Welch's correction was used to compare the vehicle, and PCB153 treated cells, with or without specific inhibitors of Erk/MAPK, PI3K/Akt and p38/MAPK pathways. One-way ANOVA, followed by Tukey's post-hoc test, was used for the multiple comparisons between three or more groups. A p-value of <0.05 was considered statistically significant.

3. Results.

3.1. PCB153 reduced apoptosis in the pituitary through the intrinsic and extrinsic pathways

Our previous results showed that the non-dioxin-like PCB153 reduced apoptosis in primary cultures of murine pituitary cells by both the intrinsic and extrinsic pathways (Raggi et al., 2016). To confirm our observations, we tested the expression and activity of the executioner caspase-3 and the initiator caspases -8 and -9 in pituitary primary cell cultures which had or had not been treated with PCB153 for 24 hours. We observed that the treatment with PCB153 significantly decreased the expression of caspase -8, -9, and -3, evaluated both at mRNA and protein levels, in comparison with the vehicle-treated pituitary cells (Figs. 1A and 1B). In addition, the caspase activity assays showed a concordant reduction in the activity of the three explored caspases in the pituitary cells treated with PCB153 (Fig 1C). These results are in agreement with the hypothesis of the anti-apoptotic effect of PCB153 in the murine pituitary. To confirm data obtained in primary cell cultures of the pituitary, the effects of PCB153 on the expression of caspases has also been explored in AtT20 and GH3 cells, two well-characterised rodent models of corticotrophs and somatotrophs cells adenoma. These experiments, overall confirmed that the treatment with PCB153 reduced the expression of caspases both at mRNA and protein levels (Supplementary Figures 2 and 3).

3.2. PCB153 reduced the expression of factors involved in the death receptor pathway

The extrinsic pathway of apoptosis has been implicated in the plastic response of the pituitary to physiological stimuli (Jaita et al., 2011) and has been suggested as a potential target to increase cell death in cellular models of pituitary adenomas (Bogazzi et al., 2004; Chen et al., 2008). To clarify the molecular mechanisms by which PCB153 reduces the activation of caspase 8 in the murine pituitary, we evaluated the variations in the expression of several molecules involved in the death receptor pathway in response to 24h treatment with PCB153 10 μM (Fig 2A). The anti-apoptotic phenotype observed after exposure to PCB153 was characterised by a significant reduction in the expression of the ligands for death receptors TNFα and FAS-L as well of the downstream adaptor proteins TRADD and FADD. In contrast, the expression of the anti-apoptotic proteins $cFLIP_{S/L}$ and IAP1/2 increased in response to the treatment with PCB153 in the primary cultures of murine hypophyseal cells. However, no differences were measured in the expression of the TNF receptor-ligand TRAIL. The downregulation of death receptor signalling was further confirmed by the co-immunoprecipitation assay which showed a reduction in the formation of FADD/pro-caspase 8 complex in our in vitro model of the pituitary exposed to PCB153 (Fig. 2B).

Taken together, our data confirm the role of PCB153 in the reduction of the activation of apoptosis which seemed to involve TNFα and FAS-L signalling and the intracellular modulation of death receptor pathway mediated by $cFLIP_{S/L}$ and IAP1/1.

3.3. Effect of PCB153 on the mitochondrial pathway of apoptosis

The intrinsic pathway of apoptosis has been related to pituitary tumorigenesis (Sambaziotis et al., 2003) and to the plastic response of the gland to physiological stimuli (Ahlbom et al., 1998). To study the effects of PCB153 in the intrinsic pathway of apoptosis, we evaluated the expression of APAF-1, cytochrome C, and proteins of the Bcl-2 family by Western Blot (Fig. 3A). In agreement with the reduction in the expression and activity of caspase 9, we observed

that PCB153 exposure was associated with a decrease in the expression of the pro-apoptotic protein APAF-1, cytochrome C, Bax, and t-Bid. In contrast, the treatment with PCB153 was accompanied by an increased expression of the anti-apoptotic molecules Bcl-2, and Bcl-XL in murine pituitary cells. We also explore the effects of PCB153 on mitochondrial translocation of Cytochrome C. Through confocal imaging, we analysed the degree of co-localisation of the Cytochrome C into mitocondria. These experiments indicate that the treatment with PCB153 was associated with a significant retention of the Cytochrome C into mitochondria of pituitary cells, if compared to controls (Fig. 3B).

Overall, our data confirm the role of PCB153 in the reduction of the intrinsic apoptotic pathway activation. In addition, the decrease in the expression of the activated Bid, t-Bid, suggests a cross-talk between the extrinsic and the intrinsic pathways of apoptosis activated by PCB153.

3.4. Anti-apoptotic and proliferative signal transduction pathways modulated by PCB153 in the pituitary

To further explore the cellular mechanisms by which PCB153 influences apoptosis of murine pituitary cells, we evaluated the expression of key molecules involved in cell fate signalling, in response to PCB153 10 uM. As shown in Figure 4A, the expression of PI3K and both the p-Akt/Akt and p-Erk/Erk ratios all increased after 24h exposure to PCB153 in comparison with the vehicle-treated cells. On the other hand, PCB153 treatment significantly reduced the protein expression of p-p38/p38-MAPK, a pathway involved in the induction of apoptosis in response to stress and DNA damage. To assess the role of these pathways in the reduction of pituitary apoptosis by PCB153 we tested the expression of activated caspase-3 with or without PCB153 10 μM and a selective inhibitor of PI3K/Akt, Erk/MAPK, and p38/MAPK (PI3K/Akt: Wortmannin, W; Erk/MAPK: PD9859, PD; p38/MAPK: SB203580, SB). Only the treatment with the inhibitor of PI3K/Akt was associated with a reverse in the suppression of caspase-3

expression induced by PCB153. On the other hand, no differences were observed following the treatment with PD and SB (Fig. 4B).

Our data suggest that PCB153 exerts its effects in the pituitary through the activation of the proliferative pathways PI3K/Akt and Erk/MAPK as well as through the simultaneous inactivation of the pro-apoptotic p38-MAPK. However, the results of the inhibition assays suggest that only the PI3K/Akt pathway plays a significant role in the apoptosis control by PCB153.

3.5. PCB153 decreases p21/p53 expression in the pituitary

The treatment of primary pituitary cells with PCB153 10 μM also led to a significant decrease in the expression of both p21 and p53. These molecules play a critical role in the cellular response to stress by the activation of cell growth arrest, cell senescence, and apoptosis. Nutlin-3 (Nutlin) is a a small-molecule that trigger the p21/p53 pathway by antagonising MDM2. To confirm our findings, we expose pituitary cells to both PCB153 and Nutlin (Fig. 5). The experiments suggested that PCB153 may counterbalance the induction of p53 and p21 by Nutlin.

3.6. The PI3K/Akt pathway mediates PCB153 activation of NF-κB.

Previous studies performed on rat liver and BRL cells linked the anti-apoptotic effects of PCB153 to the activation of NF-κB mediated by PI3K/Akt (Liu et al., 2014). Based on these elements and the results illustrated in Section 3.4, we tested the expression of NF-κB in response to PCB153 10 μM with or without the inhibitors of PI3K/Akt, Erk/MAPKm and p38/MAPK (Fig. 6B).

Interestingly, exposure to PCB153 was associated with a significant increase in NF-κB expression, in comparison with the vehicle-treated cells. The cotreatments with PCB153 and PD or SB had similar effects on the NF-κB expression in comparison with the PCB153-only

treated cells. On the other hand, the treatment with Wortmannin counteracted the effects of PCB153 on NF-κB expression.

When activated, NF-κB translocates from the cytosol to the nucleus to exert its genomic effects. To evaluate the degree of nuclear translocation of NF-κB, we used a semi-quantitative immunofluorescent approach (Noursadeghi et al., 2008) (Fig. 6A). PCB153 treatment was associated with a significant increase in NF-κB nuclear translocation. The cotreatments with PCB153 and PD or SB did not seem to influence the localisation pattern of NF-κB. However, the cotreatment with Wortmannin significantly abolished the PCB153 related NF-κB activation, as indicated by the similar pattern of NF-κB nuclear translocation in comparison with the controls (Fig. 5C). These data suggest that the PI3K/Akt pathway is involved in the increased expression and activation of NF-κB observed following PCB153 treatment, thus supporting the notion that PCB153 modulates pituitary apoptosis through an interplay between PI3K/Akt and NF-κB.

4. Discussion

PCB153 is a persistent contaminant that is ubiquitous in the environment as well as in animal and human samples despite being banned globally in 2001.

Several studies have explored the consequences of PCB153 exposure on human health with a particular focus on reproduction, development and endocrine function. Among its adverse influences, in epidemiological studies PCB153 has also been associated with proliferative and anti-apoptotic effects in cellular models and with an increased risk of some types of cancers (Abella et al., 2015; Ferrante et al., 2011; Ghisari and Bonefeld-Jorgensen, 2005; Liu et al., 2014; Ptak et al., 2011; Sánchez-Alonso et al., 2003; Tharappel et al., 2002).

The impact of exposure to PCB153 in the regulation of the proliferative and apoptotic mechanisms has been explored in various models, with discrepant results that could be related

to differences in cell types and experimental design (Abella et al., 2015; Dickerson et al., 2011; Ghosh et al., 2010). Similarly, the role of PCB153 in the modulation of cell fate in the pituitary is uncertain. Johansson *et al.* found that high doses (75 μM) of PCB153 induced necrosis in AtT20 cells without affecting apoptosis (Johansson et al., 2006). In contrast, our group observed that lower doses of the congener (10 μM) were associated with a significant decrease in the apoptotic rate of primary cells of the murine pituitary (Raggi et al., 2016).

The data described in the paper are in line with our earlier observations, confirming that acute exposure to low doses of PCB153 has anti-apoptotic effects on the murine pituitary and that this congener orchestrates both the extrinsic and the mitochondrial pathways. We found that treatment with PCB153 modulates the apoptotic machinery both at the functional and transcriptional levels, suggesting that PCB153 exposure can reduce the sensitivity of pituitary cells to proapoptotic stimuli, creating an anti-apoptotic cellular milieu. The data obtained in AtT20 and GH3 are in line with those observed in not transformed pituitary cells. The discrepant results obtained in the study and those observed in AtT20 cells by Johansson et al. may reflect the different dose of PCB153 used in the study (10 versus 75 μM) as well as in the experimental setting (Johansson et al., 2006).

We also explored the signalling pathways involved in the pituitary response to PCB153, showing that the congener activates the PI3K/Akt and Erk1/2 cascades and reduces the level of p38-MAPK and p53/p21. PI3K/Akt and Erk1/2 pathways play a crucial role in the integration of extracellular and intracellular signals and help promote cell growth and proliferation. Changes in these pathways have been observed in several tumours and are involved in the process of cancer resistance to drugs (Zheng et al., 2017). In the pituitary, PI3K/Akt and Erk pathways exert lineage-dependent effects on proliferation, differentiation and hormonal secretion (Roof and Gutierrez-Hartmann, 2018). PCB153 has been shown to activate PI3K/Akt and Erk1/2-MAPK pathways in the thyroid and the liver (Liu et al., 2015, 2014). In addition, the activation of these pathways mediates the anti-apoptotic effects of

PCB153 on hepatocytes (Liu et al., 2014). Our data are in agreement with these reports, showing that the PI3K/Akt and Erk1/2-MAPK pathways are activated in pituitary cells in response to PCB153.

p38-MAPK is responsive to extracellular stressors and is involved in the modulation of cell cycle and apoptosis. Only one study has explored the impact of PCB153 on the p38 pathway, showing that PCB153 did not change the expression of p38 and p-p38 in the rat hypothalamus (Liu et al., 2012). In contrast, we observed a reduction in the p38 expression and p-p38/p38 ratio following treatment with PCB153 in agreement with the hypothesis of a proliferative action of the congener in the pituitary. However, the cotreatment with PCB153 and SB, a specific inhibitor of p38, did not influence the caspase 3 expression in the pituitary. More studies are necessary to clarify the role of p38 in the PCB153-dependent modulation of the pituitary apoptosis.

p53 is a well-characterised tumour suppressor protein which influences cell cycle, genomic stability, and apoptosis. Low levels and specific polymorphisms of p53 have been associated with increased aggressiveness of pituitary adenomas; however, a p53 loss of function mutation is uncommon in pituitary neoplasms (Di Ieva et al., 2014; Yagnik et al., 2017). In a hepatocarcinoma cell line model, PCB153 has been related to a reduction in apoptosis due to an alteration in p53 nuclear translocation (Al-Anati et al., 2014).

p21 is a cyclin-dependent kinase inhibitor that is able to inhibit all cyclin/CDK complexes (especially cyclin E/CDK2). p21 is the primary mediator of the premature proliferative arrest induced by p53. The activation of the p53/p21 pathway is one of the mechanisms of the host defence to cancers (Mijit et al., 2020). Depending on the cell context, the activation of the p53/p21 pathway can lead to senescence or apoptosis. In the pituitary, the p53/p21 pathway has been associated with the low aggressivity and the senescence phenotype of GH-secreting adenomas (Chesnokova et al., 2013, 2008).

We observed that treatment with PCB153 is associated with a reduction in p53 and p21 expression in the pituitary. Interestingly, PCB153 seems able to counterbalance the induction of p53 and p21 dependent to Nutlin-3. These observations are in line with the hypothesis of the proliferative and anti-apoptotic effect of PCB153. More studies are necessary to assess the significance and the pathways by which PCB153 influences the p53/p21 pathway in the pituitary.

NF-κB is a fundamental transcription factor involved in cell survival and proliferation processes (Zhang et al., 2017). It is well known that the activation of NF-κB promotes cell proliferation and inhibits apoptosis, through the transcriptional activation of several anti-apoptotic genes, such as cIAP, TRAF and Bcl-2 homologue A1/Bfl-1 (Yamamoto and Gaynor, 2001). Conversely, NF-κB inhibition is related to increased apoptosis in different cellular models (Bangaru et al., 2010; Meng et al., 2012). Aberrant activation of NF- κB has been documented in different cancers. In the pituitary, NF- κB activation may play a role in the immune escape and tumour progression as suggested in cellular models as well as histological studies in humans (Chen et al., 2015).

PCB153 can activate NF-κB in the liver (Glauert et al., 2008; Tharappel et al., 2002) and adipocytes (Wu et al., 2017). PCB153 has also been previously associated with an increased nuclear localisation of NF-κB in the liver and small intestine in mouse models (Liu et al., 2014; Phillips et al., 2018). Finally, mice that do not express a p50-NF-κB subunit show a reduced hepatocarcinogenic response to PCB153 exposure (Glauert et al., 2008).

The transcriptional activity of NF-κB is regulated by several intracellular cascades, including PI3K/Akt, Erk/MAPK and p38/MAPK. The PI3K/Akt and Erk/MAPK pathways influence NF-κB action by different mechanisms. The delicate balance between the relative activation of these pathways seems to be crucial for the expression of survival and proliferative genes (Hsiung et al., 2005; Kang et al., 2011). In primary pituitary cultures, we found an increase in the expression and nuclear translocation of NF-κB following treatment with PCB153. In addition, using

specific inhibitors for PI3K/Akt, Erk1/2-MAPK and p38-MAPK pathways, we demonstrated that the activation of NF-κB promoted by PCB153 treatment is dependent on the PI3K/Akt pathway. This effect seems to be shared by other PCB congeners such as the dioxin-like PCB77, which promotes NF-κB binding to DNA via the SRC/PI3K/Akt pathway in endothelial cells (Lim et al., 2007).

The results of our study suggest the potential involvement of PCB153 in the induction and progression of pituitary tumours. However, no epidemiological research has shown a definite link between exposure to PCB153 or other congeners and the occurrence of pituitary neoplasia. Some authors have related environmental pollution to the presence of pituitary adenomas (Cannavo et al., 2010). Nevertheless, there are few data on the impact of PCBs. For instance, Cannavò et al. did not provide data on PCB levels in a survey on the effects of environmental pollution on the prevalence of acromegaly in the province of Messina (Cannavo et al., 2010). More recently, epidemiological studies, performed in an area around a site of previous PCB production, failed to demonstrate an association between PCB levels and the presence of endocrine disorders (Raffetti et al., 2018, 2017).

Data supporting the potential PCBs role in pituitary carcinogenesis come from a population exposed to high levels of dioxin and other PCBs following the Seveso accident in 1976. Twenty years after the event, a tendency toward an increased risk of pituitary adenomas was observed in the region with the maximal degree of exposure to PCBs (Pesatori et al., 2008). Interestingly, the levels of PCB153 remained high even 30 years after the accident (Warner et al., 2005), thus suggesting that this congener may contribute to the occurrence of pituitary neoplasia observed by epidemiological studies. Further investigations are needed to assess the long-term incidence of pituitary neoplasia in these subjects.

Our study has some limitations that need to be considered when interpreting the results. First of all, our observations obtained in vitro by primary cell cultures of the gland cannot be automatically seen as being representative of the in vivo impact of PCB153 on the pituitary.

This is true also for the evidence that acute and chronic exposure to PCBs can have divergent effects in terms of the proliferation and modulation of apoptosis in the liver (Liu et al., 2014; Tharappel et al., 2002). Further studies using animals treated with PCB153 are necessary to extend the results of our experiment *in vivo* or *ex vivo*.

Secondly, we used a cellular model of the complete pituitary because it allows a general panorama of the PCB153 exposure in the healthy gland. This model had the limitation that the results can not be automatically extended our results to the different cell populations that physiologically coexist in the pituitary. However, the data obtained in AtT20 and GH3, two well-established models of corticotroph and somatotroph adenoma cells, are in agreement with those observed in the whole pituitary cell culture of the mouse.

A third limit of our work is that we had tested only pituitary tissues from rodents. Therefore, our results can not be automatically extended to the human pituitary. Further study, using primary cell cultures of healthy or adenomatous human pituitary are required to extend our results to human pathology. Similarly, epidemiological toxicology studies are necessary to support the hypothesis of the role of this PCBs in the development and progression of pituitary neoplasms in humans.

Finally, in real life, PCBs exist as a complex mixture of multiple congeners, with specific chemical properties and potentially different and discordant biological effects. Because of the mechanist nature of our study, we focused only on PCB153 using exposure levels that could mimic the levels founds in biological fluids.

6. Conclusions.

The study explored the molecular pathways by which PCB153 modulates apoptosis in an in vitro model of the murine pituitary. Our results confirm that low doses of PCB153 (10 μM) reduce apoptosis through both the extrinsic and intrinsic pathways. Exposure to PCB153

activates the PI3K/Akt and Erk1/2 pathways and lowers the level of p38-MAPK and p53/21. The selective inhibition of these pathways suggests that PI3K/Akt mediates the anti-apoptotic effects of PCB153. Lastly, we showed that PI3k/Akt modulates the activation of NF-$\varkappa$B in the pituitary in response to PCB153.

Further studies are needed to clarify how PCB153 acts in the pituitary and to explore the potential connection between the inhibition of apoptosis induced by PCB153 and pituitary tumorigenesis.

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