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Hepatic signalling disruption by pollutant Polychlorinated biphenyls in steatohepatitis

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ABSTRACT

Background: Polychlorinated biphenyl-mediated steatohepatitis has been shown to be due in part to inhibition of epidermal growth factor receptor (EGFR) signalling. EGFR signalling regulates many facets of hepatocyte function, but it is unclear which other kinases and pathways are involved in the development of toxicant-associated steatohepatitis (TASH).

Methods: Comparative hepatic phosphoproteomic analysis was used to identify which kinases were affected by either PCB exposure (Aroclor 1260 mixture), high fat diet (HFD), or their interaction in a chronic exposure model of TASH. Cellular assays and western blot analysis were used to validate the phosphoproteomic findings.

Results: 1760 unique phosphorylated peptides were identified and of those 588 were significantly different. PCB exposure and dietary interaction promoted a near 25% reduction of hepatic phospho-peptides. Leptin and insulin signalling were pathways highly affected by PCB exposure and liver necrosis was a pathologic ontology over represented due to interaction between PCBs and a HFD. Casein kinase 2 (CK2), Extracellular regulated kinase (ERK), Protein kinase B (AKT), and Cyclin dependent kinase (CDK) activity were demonstrated to be down-regulated after PCB exposure and this downregulation was exacerbated with a HFD. PCB exposure led to a loss of hepatic CK2 subunit expression limiting CK2 kinase activity and negatively regulating caspase-3 (CASP3). PCBs promoted secondary necrosis *in vitro* validating the latter observation. The loss of hepatic phosphoprotein signalling appeared to be due to decreased signal transduction rather than phosphatase upregulation.

Conclusions: PCBs are signal disrupting chemicals that promote secondary necrosis through affecting a myriad of liver processes including metabolism and cellular maintenance. PCB exposure, particularly with interaction with a HFD greatly down-regulates the hepatic kinome. More data are needed on signalling disruption and its impact on liver health.

Abbreviations: AKT, Serine/Threonine kinase 1; CAR, Constitutive androstane receptor; CASP3, Caspase-3; CD, Control diet; CD+, Control diet + Aroclor 1260 exposure; CDK, Cyclin dependent kinase; CK2, Casein kinase 2; CK-18, Cytokeratin-18; EGFR, Epidermal growth factor receptor; EDCs, Endocrine disrupting chemicals; ERK, Extracellular regulated kinase; FASP, Filter aided sample prep; FDR, False discovery rate; FXR, Farnesoid X receptor; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; HFD, High fat diet; HFD+, high fat diet + Aroclor 1260 exposure; HNF4α, Hepatocyte nuclear factor receptor alpha; LXR, Liver X receptor; MDCs, Metabolic disrupting chemicals; NAFLD, Nonalcoholic fatty liver disease; NHANES, National Health and Nutrition Examination Survey; NRF2, Nuclear factor (erythroid-derived 2)-like 2; PCBs, Polychlorinated biphenyls; PI3K, Phosphoinositide 3-kinase; PP2α, Serine/Threonine protein phosphatase 2a; PTP1B, Protein-tyrosine phosphatase 1B; SDCs, Signalling disrupting chemicals; TASH, Toxicant associated steatohepatitis

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1. Introduction

Polychlorinated biphenyls (PCBs) are endocrine, metabolic, and signalling disrupting chemicals (EDCs, MDCs, SDCs) [1,2]. PCBs still persist in the environment even though their production has long been banned. The primary route of human exposure is through consumption of contaminated food leading to bioaccumulation [3]. The global prevalence of non-alcoholic fatty liver disease (NAFLD) is currently at 25% and cannot be fully explained by dietary, genetic, or level of physical activity [4]. Chemical pollutant exposures may be an understudied pathogenic factor in this epidemic of fatty liver disease. Metabolic effects due to PCBs are of great concern since 100% of National Health and Nutrition Examination Survey (NHANES) participants had detectable serum levels of PCBs [5]. PCBs were associated with increased odds ratios for unexplained ALT elevation, a surrogate biomarker for NAFLD. The Anniston Community Health Survey (ACHS) is a highly exposed cohort of residents living near a former PCB manufacturing plant in Alabama. Elevated PCB body burden in this cohort was positively associated with suspected TASH and the liver necrosis marker cytokeratin 18 (KRT18) M65 [6]. In animal models that mimic these human exposures, the development of PCB-mediated TASH was contingent upon both the exposure and the feeding of a high fat diet (HFD). PCB exposure has been shown to elicit a transcriptional response of xenobiotic metabolizing genes mediated through the constitutive androstane receptor (CAR) [7–10]. PCBs were considered direct CAR agonists, but recent studies have demonstrated that they may also act through epidermal growth factor receptor (EGFR) inhibition leading to indirect CAR activation [1,11]. EGFR is a receptor tyrosine kinase that is highly expressed in the liver but little is known about its physiological role in liver homeostasis and metabolism [12]. EGFR can regulate the phosphorylation state of many downstream targets including, but not limited to CAR, hepatocyte nuclear factor 4 alpha (HNF4α), and nuclear factor (erythroid-derived 2)-like 2 (NRF2) [13]. Deficits in cell signalling have pathological relevance in metabolic disease with loss of insulin signalling and diabetes being the strongest example [14]. Loss of other signalling pathways in the context of other metabolic diseases like steatohepatitis are understudied and may be just as relevant. Kinase inhibition is closely associated with cell death, metabolic disruption, and possibly steatohepatitis. To characterize the complexity of PCB-mediated signalling disruption in the liver, a phosphoproteomic approach was used. In this study, we aimed to characterize the dietary and PCB-mediated effects on the hepatic phosphoproteome in an animal model of TASH and determine how PCBs promote hepatocyte necrosis.

2. Materials and methods

2.1. Animal studies

The C57Bl/6 (male) mouse liver samples used in this study were obtained from archived (−80 °C) tissues from a previous study [15]. This study used a protocol approved by the University of Louisville Institutional Animal Care and Use Committee. Guidelines provided by the NIH guide for the care and use of laboratory animals were abided by for this research. In this study, three samples per group were randomly selected and used for the proteomic study. Mice fed a HFD developed steatosis and mice fed a control diet and treated with Aroclor 1260 did not develop fatty liver. The mice fed a HFD and treated with Aroclor 1260 developed steatohepatitis [15]. The following abbreviations in the figures represent the animal groups; control diet (CD), control diet with Aroclor 1260 exposure (CD+), high fat diet (HFD), high fat diet with Aroclor exposure (HFD+).

2.2. Phospho-peptide sample preparation

Mouse liver tissue were homogenized in 1% SDS RIPA buffer. Protein lysate (100 μg) per sample was prepared and trypsinized using the filter aided sample prep (FASP) protocol [16]. Phosphopeptides were then further enriched with TiO_2 and purified using C18 columns [17]. Peptide concentrations were measured by measuring absorbance at 205 nm to determine the recovery amount to use for subsequent liquid chromatography and mass spectrometry analysis.

2.3. Liquid chromatography for separation of phospho-peptides

The columns used were an Acclaim PepMap 100 75 μm × 2 cm, nanoViper (C18, 3 μm, 100 Å) trap, and an Acclaim PepMap RSLC 50 μm × 15 cm, nanoViper (C18, 2 μm, 100 Å) separating column (ThermoFisher Scientific, Waltham, MA, USA). An EASY-nLC 1000 UHPLC system (ThermoFisher, Waltham, MA) was used with solvents A = 2% *v*/v acetonitrile / 0.1% v/v formic acid and B = 80% v/v acetonitrile / 0.1% v/v formic acid as mobile phases. Following injection of 500 ng of sample onto the trap, separation was accomplished at 300 nL/min with a 110-min linear gradient from 0% B to 50% B, followed by a 5 min linear gradient from 50% B to 95% B, and lastly a 5-min wash with 95% B. A 40 mm stainless steel emitter (ThermoFisher, Waltham, MA) was coupled to the outlet of the separating column. A Nanospray Flex source (ThermoFisher, Waltham, MA) was used to position the end of the emitter near the ion transfer capillary of the mass spectrometer. The ion transfer capillary temperature of the mass spectrometer was set at 225 °C, and the spray voltage was set at 1.6 kV.

2.4. Mass spectrometry

An Orbitrap Elite – ETD mass spectrometer (ThermoFisher) was used to collect data from the LC eluate. An Nth Order Double Play was created in Xcalibur v2.2. Scan event one of the methods obtained an FTMS MS1 scan (normal mass range; 240,000 resolution, full scan type, positive polarity, profile data type) for the range 300-2000 *m/z*. Scan event two obtained ITMS MS2 scans (normal mass range, rapid scan rate, centroid data type) on up to twenty peaks that had a minimum signal threshold of 5000 counts from scan event one. The lock mass option was enabled (0% lock mass abundance) using the 371.101236 m/z polysiloxane peak as an internal calibrant.

2.5. Data analysis with peaks studio 7.5

The raw data files were analyzed separately with Peaks Studio 7.5 using the UniprotKB mouse reviewed canonical and isoform protein sequences current as of 3/21/2017 and the Denovo, PeaksDB, and PeaksPTM algorithms. Identifications at the 1% FDR threshold from the PeaksDB and PeaksPTM results were loaded into the Peaks Label Free Quantification algorithm. Data files for acquired LCMS data (.RAW), search engine files (.mgf), peak list files (.mzML) files, and search results aggregated into a Scaffold3 (.sf3, ProteomeSoftware.com) will are deposited with MassIVE (http://massive.ucsd.edu/) data repository with the Center for Computational Mass Spectrometry at the University of California, San Diego and shared with the ProteomeXchange (www.proteomexchange.org).

2.6. MetaCore analysis

Phosphorylated proteins with significantly different abundances ($p < .05$) due to a HFD, Aroclor exposure, or interaction were analyzed with MetaCore pathway, processes, and pathology ontologies. Only pathways, processes, and pathologies identified with a false discovery rate (FDR) less than or equal to 0.05 were accepted.

2.7. PhosphoScanSite analysis

Phosphosites were searched using the Massachusetts Institute of

Technology's free software PhosphoScansite 3 [18]. Low, medium, and high stringency thresholds were used to identify potential kinases regulating differentially expressed phospho-substrates.

3. Cell Culture

Cryopreserved human hepatocytes obtained from BioreclamationIVT supplemented with Invitrogro Hepatocyte Media and Torpedo antibiotic solution (BioreclamationIVT, Westbury, NY). The cells were plated on collagen plates and incubated in a 5% carbon dioxide atmosphere and 95% humidity at 37 °C.

4. Cellomic Cell Death Assay

Cryopreserved human hepatocytes were exposed to either 0.5% DMSO (DMSO applied to cryopreserved human hepatocytes is not toxic up to 2% [19]) or Aroclor 1260 (10 μg/mL) for 6 and 24 h. At each time point cells were stained with Hoechst 33342 (Thermo-Scientific, Waltham, MA) and imaged. Data for DNA fragmentation percent (Cells below 2 N DNA content) and nuclear area were taken at each time point. Apoptosis and necrosis demonstrate DNA fragmentation but apoptotic nuclei are smaller, thus enabling these two factors to be used to distinguish between live, apoptotic, and necrotic cells [20].

5. Western blot analysis

Mouse liver lysates were homogenized in RIPA Buffer (100 mg tissue/0.5 mL RIPA supplemented with protease, and phosphatase inhibitors 10 μL/mL, (Sigma-Aldrich, St. Louis, MO)). The protein concentration was determined by the bicinchoninic acid protein assay (Sigma-Aldrich, St. Louis, MO). Protein (15 μg) was separated on 7.5% SDS Gel (BioRad, Hercules, CA), transferred to polyvinylidene difluoride membranes and blocked, incubated with primary and then secondary antibodies. Western blot protein bands were quantified using BioRad Image Software. The following primary antibodies were used: CK2α, CK2 phosphosubstrate, PP2α, and CASP3 (Cell Signalling Technology, Danvers, MA); CK2β, PTP1B, S150 CASP3 (abcam Cambridge, UK).

5.1. Statistical analysis

Western blot densitometry values using GraphPad Prism Version 7 for Macintosh (San Diego, CA). The data are expressed as box and whisker plots for western blot analysis ($n = 5$) and mean ± SEM for the Cellomic data ($n = 24$). Data was compared using two-way ANOVA for western blot analysis. $P < .05$ was considered statistically significant. Quantified phospho-peptides from MS data were statistically compared by two-way ANOVA with a looping two-way ANOVA script in R software package. The following assumptions were verified for use of the two-way ANOVA, 2 independent variables, continuous dependent variable, categorical independent variables, sample independence, variance equality, and normality [21].

6. Results

6.1. Phosphoproteomic analysis identifies phosphorylated substrates altered by a HFD, Aroclor exposure, and their interaction

An unbiased phosphoproteomic approach was implemented to identify the effects of diet and PCBs on the hepatic phosphoproteome. Fig. 1A Illustrates the phosphoproteomic workflow and analysis of murine liver from control diet (CD) fed mice or HFD fed mice with or without 12-week exposure to Aroclor 1260 (20 mg/kg). 1760 unique phospho-peptides were identified in this study of which 588 were significant. Fig. 1B demonstrates that 113 phospho-peptides were significantly increased, while 104 were decreased due to a high fat diet. Aroclor 1260 exposure decreased levels of 131 phospho peptides and increased 46 (Fig. 1C). The interaction between a HFD and Aroclor

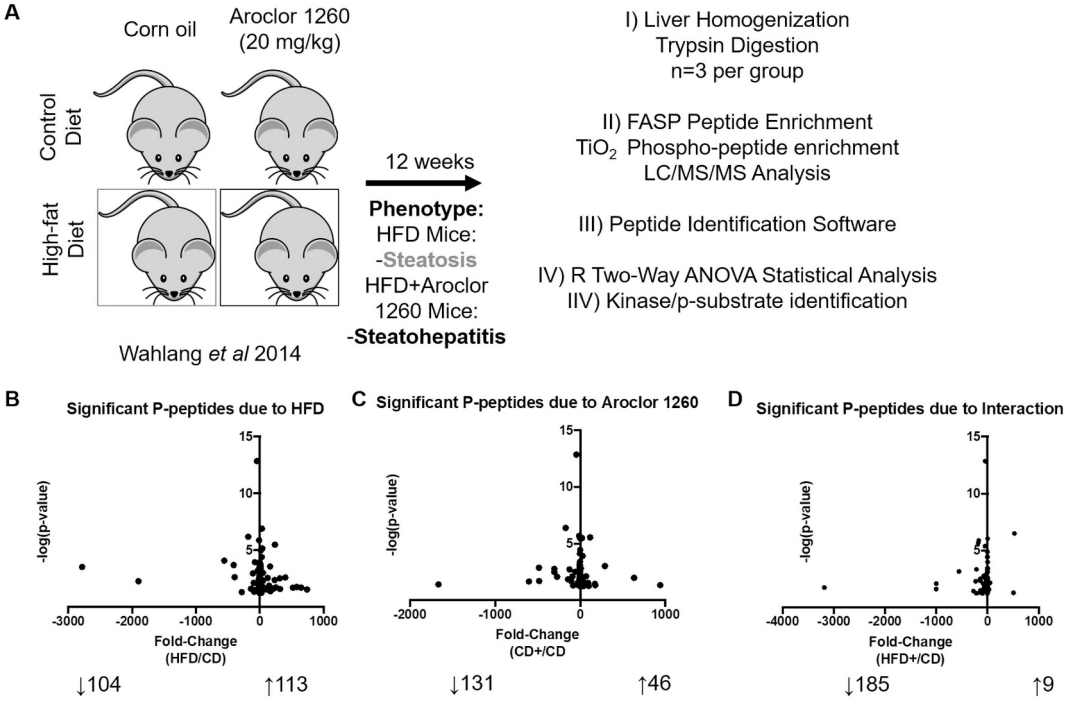


Fig. 1. Phosphoproteomic analysis identifies phosphorylated substrates altered by Aroclor 1260 exposure, a HFD, and their interaction A. Phosphoproteomic workflow is illustrated. B-D. Volcano plots of significant p-peptides fold-change *vs.* -log(*p*-value) for HFD (HFD/CD fold-change), Aroclor (CD + /CD fold-change), and their interaction (H + /CD fold-change). B. A HFD enriched 113 p-peptides and diminished 104 p-peptides. C. Aroclor 1260 exposure enriched 46 p-peptides and diminished 131. D. The interaction of the two variables enriched 9 p-peptides and diminished 185. Phospho-peptide abundances were compared by two-way ANOVA and only peptides that were significantly changed ($P < .05$) were investigated further.

exposure decreased 185 phospho peptides and increased only 9. Remarkably, nearly 25% of hepatic phospho-peptides were significantly decreased by either PCB or PCB-HFD interaction (Fig. 1D). These data suggest that PCBs inhibit phosphoprotein signalling, consistent with our previous studies [1,11]. However, the degree of loss of phosphoprotein signalling due to PCBs was not anticipated.

6.2. MetaCore pathway, processes, and pathology analysis of significant peptides

Proteins with significant different abundances of phosphosites due to a HFD, Aroclor exposure, or their interaction were analyzed with MetaCore ontologies. Table 1A demonstrates that a HFD affected ontologies involved in lipid accumulation, adiponectin signalling, and lipoprotein assembly. Table 1B illustrates that Aroclor exposure affects pathways involved in PI3K, insulin, LXR, FXR, short chain fatty acids (SCFAs) (butanoic, propanoic acids), and Sirtuin signalling. Table 1C demonstrates that HFD and Aroclor interaction affected liver necrosis and protein chaperones.

6.3. PhosphoScanSite 3 analysis of the significant phosphopeptides demonstrate that many kinases are downregulated with Aroclor exposure and the Aroclor and HFD interaction

Using the significant p-substrates and the direction that their abundance was altered due to experimental variables, a kinome analysis was conducted. Fig. 2A demonstrates that some kinases were predicted to have higher activity, while others were lower with a HFD. Fig. 2B shows that an overwhelming majority of kinases are predicted to have lower activity with Aroclor exposure. These downregulated kinases include ERK, AKT, CK2, and CDKs. Fig. 2C demonstrates that the kinome was downregulated to an even greater extent by the interaction of a HFD and Aroclor exposure. ERK, AKT, CK2, and CDKs were downregulated due to interaction of a HFD and Aroclor exposure as well. This implicates loss of phosphoprotein signalling in the pathogenesis of TASH.

6.4. PP2α and PTP1b were not significantly upregulated with Aroclor exposure suggesting the cumulative loss of phosphoproteins is due to loss of signal rather than degradation

Since the levels of protein phosphorylation are dramatically downregulated with PCB exposure and with dietary interaction, the expression of phosphatases was assessed. Fig. 3A-B demonstrate that Ser/Thr phosphatase PP2α and Tyr phosphatase PTP1B were not significantly changed with PCB exposure or diet. This suggests that the loss of phosphoproteins and kinase activity cannot be explained by increased phosphatase levels, but loss of activation through phosphorylation.

6.5. Aroclor exposure diminishes CK2 activity and reduces expression of the α and β subunits but increases the expression of the α' subunit

Since CK2 was projected to have decreased kinase activity after Aroclor exposure, CK2 activity and the pattern of expression of its subunits were measured. CK2 can function as a tetramer and is thought to be constitutively active. Fig. 4A-D demonstrates that Aroclor exposure diminishes the protein expression of CK2α 97% ($p < .01$), and CK2β 67% ($p < .01$), and CK2 kinase activity 81% ($p < .002$) (CD *vs* CD +) but increases the expression of CK2α' ($p < .0006$). The expression of CK2α' is most pronounced in the livers of mice that developed TASH (HFD +). CK2 can function as a tetrameric enzyme with either 2 alpha subunits and 2 beta subunits or 2 alpha' subunits and two beta subunits. Interestingly PCB exposure promotes CK2 alpha' subunit expression which is more pronounced with a HFD and PCB exposure.

6.6. Aroclor exposure prevents CASP3 phosphorylation and promotes secondary necrosis in vitro

CK2 negatively regulates cell death through phosphorylation of caspases at residues surrounding their cleavage sites, thereby, preventing caspase-mediated cell death. Since TASH is distinguished by elevated hepatic cell death and there is a loss of CK2 activity with PCB exposure, caspase-3 (CASP3) phosphorylation and cell death were measured. In Fig. 5A CASP3 expression is actually reduced 59% ($p < .0003$) with PCB exposure or a HFD 63% ($p < .0001$) and its decline is more pronounced with the co-exposure (31%) ($p < .0016$) relative to CD expression. Fig. 5B demonstrates that phosphorylation of CASP3 at S150 is downregulated with Aroclor exposure (52%) ($p < .006$), HFD (49%)($p < .02$), and their interaction (40%) ($p < .0005$). Fig. 5C illustrates that primary human hepatocytes exposed to PCBs first undergo apoptosis 33.3% of cells at 6 h and 41.7% at 24 h are apoptotic, followed by 4.2% of cells undergoing secondary necrosis at 6 h, and 41.7% at 24 h (Fig. 5D).

7. Discussion

PCBs were previously characterized as EGFR antagonists *in vivo* and *in vitro* and coined "signalling disrupting chemicals" providing a rationale to investigate the hepatic phosphoproteome in an animal model of PCB-mediated TASH [1]. Cell signalling through protein phosphorylation *via* kinases is critical for cells to respond to intracellular and extracellular cues rapidly. We postulate that downregulation of phosphoproteomic signalling by PCBs could sensitize the liver to HFD-induced steatohepatitis. The most well characterized example of loss of hepatic signalling in metabolic disease is insulin resistance [14]. While the insulin receptor is one of the most well studied receptor tyrosine kinases in metabolism, the EGFR is highly expressed in the liver and is under-studied in the context of NAFLD. However, not all of the kinome

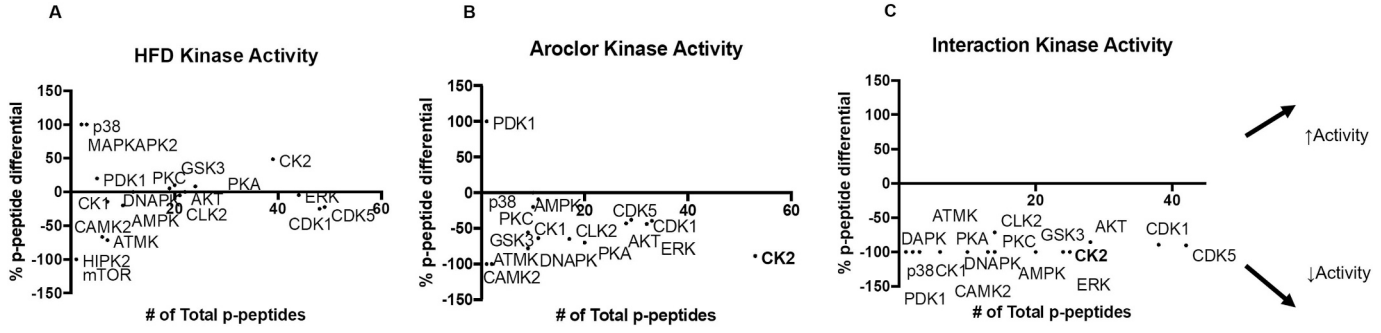


Fig. 2. PhosphoScanSite 3 analysis of the significant phosphpeptides demonstrates that many kinases are downregulated with Aroclor exposure and the Aroclor and HFD interaction A. The kinome changes predicted to be altered by a HFD, B. Aroclor exposure, C. or interaction. P-peptides that were significantly different by two-way ANOVA were used in the PhosphoScanSite 3 analysis to determine what kinase regulates the substrate.

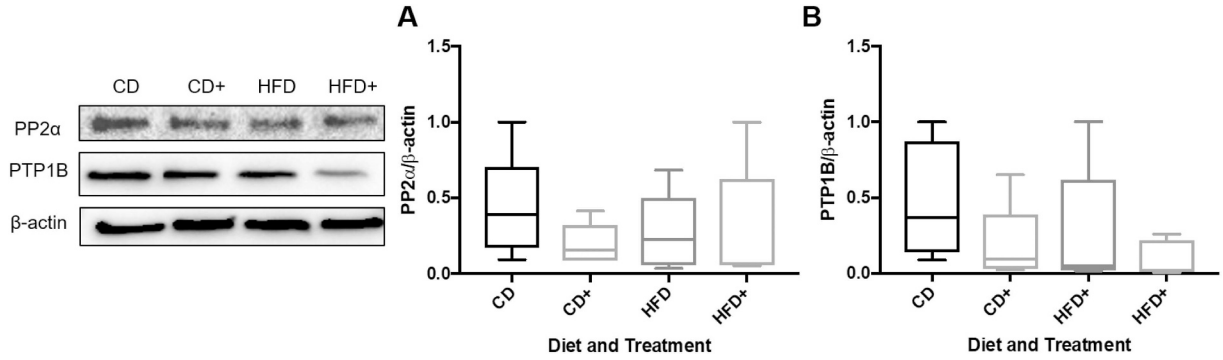


Fig. 3. PP2A and PTP1b were not significantly upregulated with Aroclor exposure suggesting the cumulative loss phosphoprotein signalling is due loss of signal and not enhanced degradation. A. Immunoblot analysis of hepatic PP2α and B. PTP1B. The following abbreviations in the graphs represent the subsequent groups; control diet (CD), control diet with Aroclor 1260 exposure (CD +), high fat diet (HFD), high fat diet with Aroclor exposure (HFD +). A $n = 5$ was used for the immunoblot analysis. A two-way ANOVA was used to statistically compare these datasets.

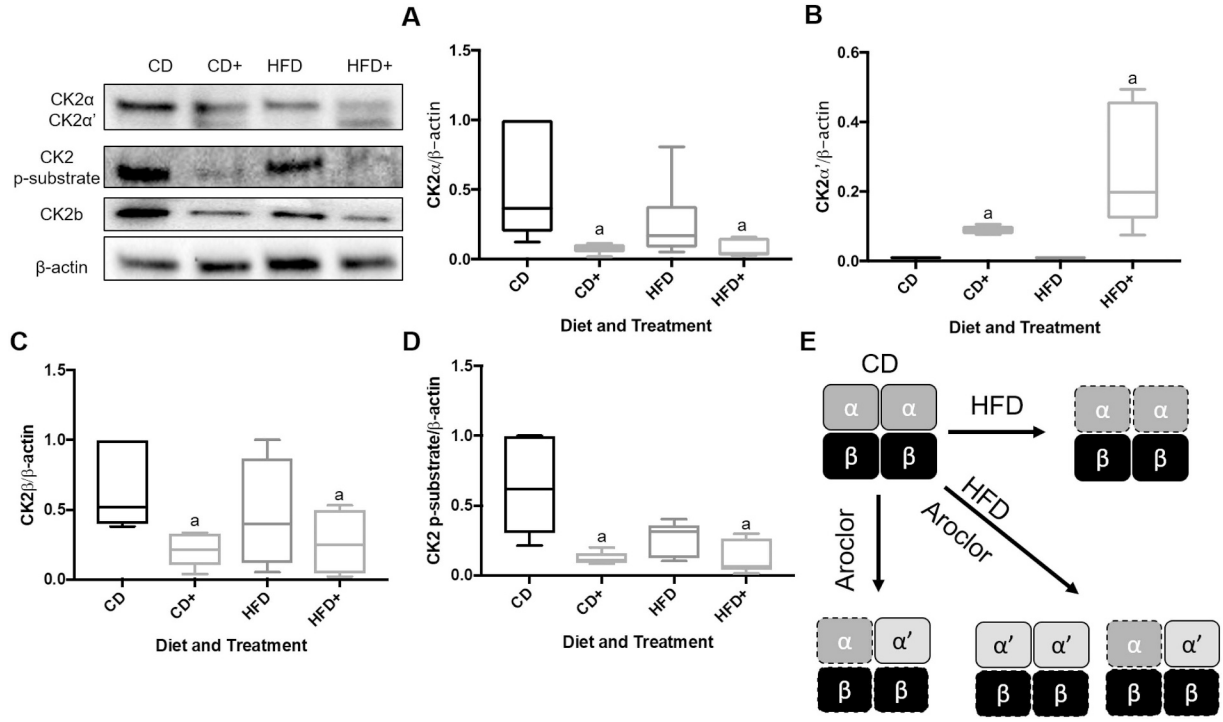
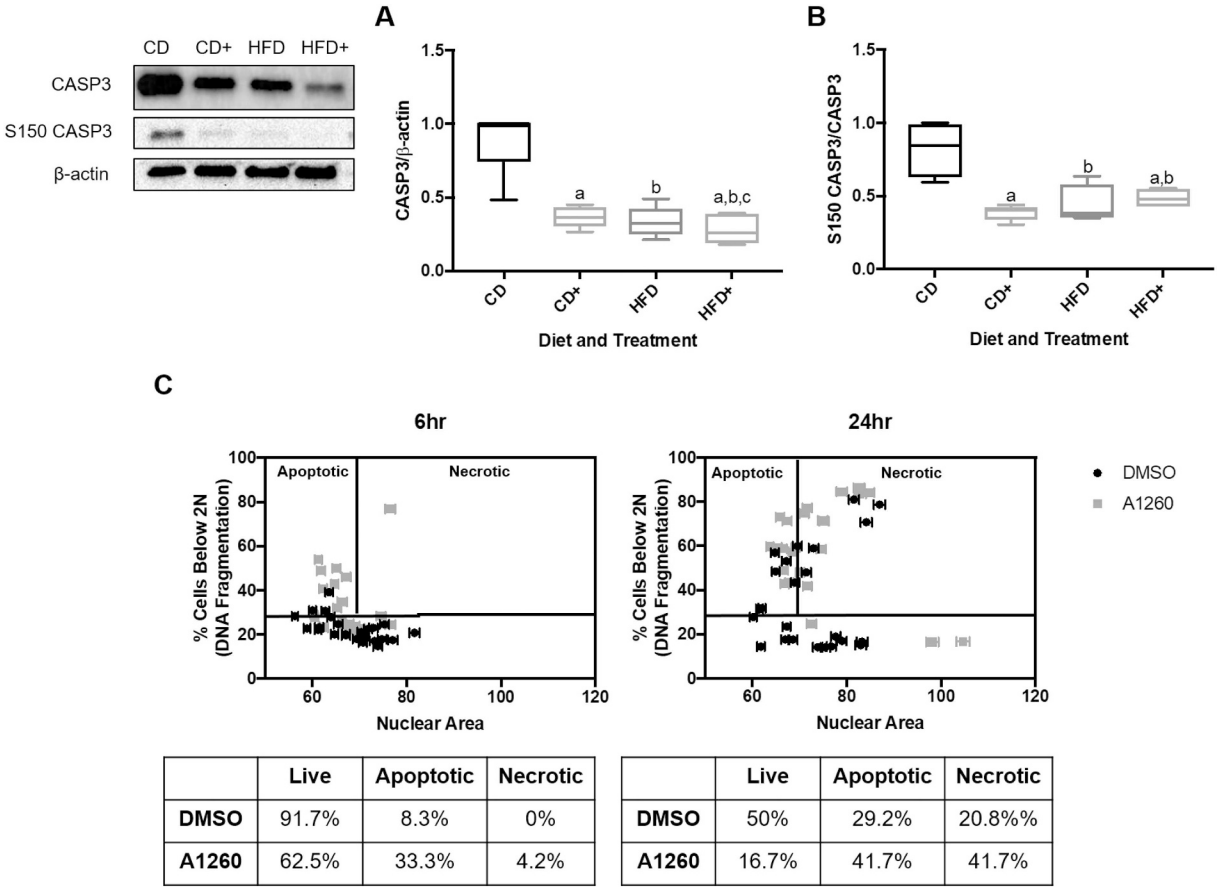


Fig. 4. Aroclor exposure diminishes CK2 activity and reduces expression of the α and β subunits but increases the expression of the α' subunit A. Immunoblot analysis of CK2α, B. CK2α', C. CK2β expression, and D. CK2 phospho-substrate. E. Illustration of alterations in CK2 subunit expression relative to CD expression. A n = 5 was used for the immunoblot analysis. The following abbreviations in the graphs represent the subsequent groups; control diet (CD), control diet with Aroclor 1260 exposure (CD +), high fat diet (HFD), high fat diet with Aroclor exposure (HFD +). A two-way ANOVA was used to statistically compare these datasets.

affected were downstream of EGFR. The manner by which PCBs decrease activity of other kinases is unknown. Previously however, loss in the phosphorylation of several of these kinases (*e.g.* EGFR, AKT, ERK, mTOR) has already been validated *via* western blot analysis [1,11]. However, while the inhibition of EGFR by PCBs was the rationale to perform these analysis the results demonstrate that loss of EGFR signalling is only part of the story.

Interestingly, a majority of the hepatic phosphopeptides were significantly decreased with Aroclor exposure and the PCB-dietary interaction. Roughly, a quarter of the phosphopeptides were diminished in the livers of mice that developed TASH suggesting loss of cell signalling could contribute to the development of TASH. When analyzing the kinome alterations due to the experiment variables, Aroclor exposure suppressed kinase activity and the Aroclor-HFD interaction exacerbated this effect. Since protein phosphorylation can be regulated by kinases and phosphatases [22], the abundance of serine/threonine/tyrosine phosphatases were determined. The decrease in phosphoproteins was not due to upregulation of either PP2α, or PTP1B. This suggests that the observed effect was due to decreased signal rather than elevated signal degradation.

Recently, PCB-126 has been shown to inhibit activation of hepatic AMPK pathway similar to our findings [23]. In addition, PCB-126 can promote liver disease in *in vivo* [24] and was demonstrated to be the most potent EGFR inhibitor (of the PCB congeners) tested *in vitro* [1]. PCB-153 worsened steatosis *in vivo* [25], and was a potent EGFR inhibitor *in vitro* as well, but less so than PCB-126. Aroclor 1254, a lower chlorinated PCB mixture, can prevent glucose metabolism *in vivo* through disruption of insulin receptor signalling [26]. These serve as examples of hepatic signalling disruption due to varied PCB congeners and mixtures. This study proposes that signalling disruption is a critical biological effect of PCB exposures that can contribute to TASH. More studies are required in the future to investigate multiple PCB congeners



	Live	Apoptotic	Necrotic
DMSO	91.7%	8.3%	0%
A1260	62.5%	33.3%	4.2%

	Live	Apoptotic	Necrotic
DMSO	50%	29.2%	20.8%%
A1260	16.7%	41.7%	41.7%

Fig. 5. Aroclor exposure promotes secondary necrosis in TASH A. Immunoblot analysis of hepatic CASP3, B. S150 CASP3. C. Cellomic cell death measure in cryopreserved human hepatocytes at 6 and 24 h. The following abbreviations in the graphs represent the subsequent groups; control diet (CD), control diet with Aroclor 1260 exposure (CD +), high fat diet (HFD), high fat diet with Aroclor exposure (HFD +).A n = 5 was used for the immunoblot analysis. A $n = 24$ was for the Cellomic assay, each plotted point is a mean ± SEM for 2500 cells. A two-way ANOVA was used to statistically compare the western blot analysis data.

and mixture effects on hepatic signalling *in vivo*, as this is a field in its infancy.

The MetaCore analysis demonstrated that a HFD affects hepatic lipid accumulation that is well established in many HFD fed animal models [27,28]. The interaction of a HFD and PCB exposure highlighted liver necrosis pathology which reinforces previous findings [25,29,30]. Aroclor was found to affect pathways involving PI3K (phosphoinositide 3-kinase), insulin and LXR (Liver X receptor) that has previously been demonstrated with PCB exposures in the liver [1,6,11]. These animals were in the fasted state where hepatic EGFR regulates PI3K activity [31]. Thus, the pathway analysis highlighting insulin and leptin signalling (fed state receptors) as pathways affected by Aroclor exposure was interesting as these receptors all overlap in their regulation of PI3K [32]. Previously elevated PCB body burden in humans was directly correlated to decreases in serum leptin and insulin [6]. This may in part explain the pathway analysis and further contribute to understanding how PCBs disrupt cell signalling through limiting the abundance of metabolic receptor ligands. In addition to insulin and leptin signalling affected by PCB exposure MetaCore analysis identified other ontologies affected by PCB exposure. For example, Aroclor 1260 impacted nuclear receptors (FXR, LXR), sirtuin protein regulation, SCFA metabolism, and acetyl CoA carboxylase (ACC). Each of these pathways have pathologic implications in the sensitization to diet induced fatty liver disease. For instance FXR agonists and ACC inhibitors are in development for the treatment of NASH [33].

The Endocrine Society defines endocrine disruptors as chemicals that interfere with any aspect of hormone action [2,34]. Thus, this present manuscript clarifies mechanisms of PCB-related endocrine disruption. Two known factors account for the decrease in signal. First, PCBs have been shown to inhibit at least one RTK, namely the EGFR [1,11]. Due to homology with other conserved receptor structure, it is possible that PCBs could also inhibit related receptors [35]. Secondly, PCBs are endocrine disruptors which have been shown to decrease circulating hormone levels [6]. For example, PCB exposures were associated with reduced insulin and leptin levels in both animal and human studies (ACHS) [6,15]. This is consistent with decreased insulin and leptin action detected by MetaCore ontologies in this study. Thus, PCBs can decrease protein hormone levels as well as protein hormone receptor function eliciting a cell signalling deficit. Mechanisms of cell death are most highly associated with the inhibition of cell signalling (*e.g.* kinase inhibitors). Kinase inhibition dependent cell death has been well studied in the cancer field but in contrast the effects on liver function are understudied [36].

One kinase not previously demonstrated to be downregulated by PCBs in the development of TASH was CK2. CK2 has been shown to negatively regulate cell death through phosphorylation of residues surrounding caspase cleavage sites, thereby, preventing cleavage [37]. CK2 is unique, in that it is made up of subunits and is considered constitutively active [38,39]. CK2 is thought to be constitutively active in order to prevent incidental caspase-mediated apoptosis through caspase phosphorylation by CK2 [39,40]. However, little is known in regard to the subunit configuration on kinase activity [38,39]. The data suggested that hepatic CK2 activity is decreased with Aroclor exposure. Our validation experiment confirmed this initial observation and demonstrated that the subunit composition is altered with PCB exposure, resulting in an inhibitory effect on kinase activity. Interestingly with

PCB exposure, the CK2a' subunit was more highly expressed, and this was further increased in mice that were exposed and fed a HFD, clearly demonstrating an interaction unique to TASH. CK2α' is a key effector kinase in interleukin-6 and tumor necrosis factor alpha signalling in inflammation which may explain the elevated expression with PCB exposure [41]. Previously cytokine elevation was associated with PCB exposures TASH animal models [15,30] and human studies (ACHS) [6]. Also CK2β is downregulated with Aroclor exposure, as well, which has been shown to be required for the tetrameric formation [38]. CK2 is the main regulator CASP3 phosphorylation that negatively regulates apoptosis through prevention of CASP3 self-cleavage and activation [37,40,42].

CASP3 is a measure of apoptotic cell death that can be due to intrinsic or extrinsic activation [37,42]. Apoptosis is a programmed form of cell death that is often considered non-inflammatory, as opposed to necrosis [43]. Recent data demonstrates that in liver disease models, apoptosis is the initial cell death mechanism observed, but once immune cell-mediated apoptotic cell clearance is saturated (or no immune cells are present as seen in *in vitro* models), secondary necrosis occurs [44]. Our data supports this model as CASP3 expression is decreased relative to control with either PCB exposure or a HFD suggesting it is undergoing post-apoptotic degradation. This is further exacerbated in the livers of mice fed a HFD and exposed to PCBs. In primary human hepatocytes exposed to Aroclor 1260, cell apoptosis is first observed at 6 h, followed by a rise in necrotic death at 24 h. Since this a rapid PCB effect it would present as necrosis *in vivo*. These findings suggest that PCB exposure promotes apoptosis followed by secondary necrosis. This adds support to human studies where elevated whole CK-18 (necrosis marker) in serum is associated with PCB load in exposed populations [6].

8. Conclusion

This is the first phosphoproteomic analysis of an animal model of TASH. Collectively, the data suggest that loss of protein phosphorylation (25% loss of phospho-peptides in TASH) and down-regulation of the hepatic kinome is a major factor in the development of PCB-mediated TASH (Graphical Abstract). This study confirms past studies where PCBs downregulated hepatic AKT, ERK, and mTOR activity [1,11]. CK2 is an additional kinase that is downregulated in TASH, due to PCB-mediated loss of CK2α and CK2β subunit expression. PCB-mediated loss of CK2 activity prevents negative phospho-regulation of CASP3. Secondary necrosis appears to be the final mechanism of cell death in PCB-mediated TASH *in vivo* and *in vitro*. Additional human model system investigation of PCB-mediated signalling disruption and NAFLD are required for the future.

Table 1: MetaCore pathway, processes, and pathology analysis of peptides affected by a HFD, Aroclor, and their interaction A. Ontologies affected by a HFD, B. Aroclor exposure, and C. interaction. Phosphopeptides that were significant by two-way ANOVA were used for this analysis and only pathways that met the FDR of 0.05 were used.

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