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## DEVELOPMENTAL CIGARETTE SMOKE EXPOSURE II: HEPATIC PROTEOME PROFILES IN 6 MONTH OLD ADULT OFFSPRING

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### Abstract

Utilizing a mouse model of ‘active’ developmental cigarette smoke exposure (CSE) [gestational day (GD) 1 through postnatal day (PD) 21] characterized by offspring low birth weight, the impact of developmental CSE on liver proteome profiles of adult offspring at 6 months of age was determined. Liver tissue was collected from Sham- and CSE-offspring for 2D-SDS-PAGE based proteome analysis with Partial Least Squares-Discriminant Analysis (PLS-DA). A similar study conducted at the cessation of exposure to cigarette smoke documented decreased gluconeogenesis coupled to oxidative stress in weanling offspring. In the current study, exposure throughout development to cigarette smoke resulted in impaired hepatic carbohydrate metabolism, decreased serum glucose levels, and increased gluconeogenic regulatory enzyme abundances during the fed-state coupled to decreased expression of SIRT1 as well as increased PEPCK and PGC1 $\alpha$  expression. Together these findings indicate inappropriately timed gluconeogenesis that may reflect impaired insulin signaling in mature offspring exposed to ‘active’ developmental CSE.

### Keywords

Cigarette smoke; liver; proteome; murine; developmental; tobacco

## 1 INTRODUCTION

Maternal environmental exposures, nutrition, and lifestyle play critical roles in fetal growth and development [1–4]. According to the Center for Disease Control and Prevention, approximately 20% of women smoke cigarettes at some time during pregnancy [5] though

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both epidemiological evidence and animal studies suggest that consumption of tobacco products during pregnancy is hazardous to the fetus [5–15]. Maternal cigarette smoke exposure during gestation is associated with a host of adverse reproductive outcomes, congenital anomalies, and a high incidence of intrauterine growth restriction and resulting infant low birth weight [5, 11, 12, 14–16]. Moreover, children manifest adverse outcomes of fetal exposure to tobacco smoke that persist into childhood such as an increased incidence of respiratory infections and asthma as well as a propensity towards infant/childhood behavioral and cognitive deficits [5, 11, 12, 14–16]. Interestingly, more recent studies have established compelling linkages between pre-/perinatal exposures to cigarette smoke and an increased risk of offspring obesity and metabolic disease [17–25]. While the ability of prenatal and early-life environmental exposures such as cigarette smoke to elicit developmental programming and long-lived alterations in adult health/disease risk has been well documented [1, 4, 26–31], the precise biological mechanisms by which such exposures dysregulate the development, function, and adaptability of the organism's key metabolic regulatory tissues/organs are still largely unknown.

Cigarette smoke contains a multitude of combustion gases, heavy metals, flavorings and other additives including addictive substances such as nicotine [32–35]; The toxins in cigarette smoke are generally transferred across the placenta to the embryo/fetus [36], exposing rapidly proliferating and differentiating cells/tissues to a mixture of over 8000 toxic substances [37]. Although a primary site of exogenous compound detoxification in adult humans [38, 39], the liver has limited capacity to detoxify such compounds in the embryo/fetus [40–42] and, as such, the developing organ is functionally challenged, in a premature state, by this myriad of toxins. The diversity of potentially toxic substances in cigarette smoke, along with multiplicity of metabolic organs that are targeted by exposure, makes it difficult to elucidate the mechanisms through which developmental exposure to cigarette smoke elicits long-lived metabolic consequences in exposed offspring. Key insights may be found by examining alterations in genomic/epigenomic transcriptomic, proteomic, and metabolomic signatures of the primary metabolic tissues between exposed and non-exposed offspring. Such strategies have been utilized successfully to delineate the mechanistic underpinnings of 'developmental reprogramming' and long-lived metabolic consequences of *in utero* and early-life xenobiotic exposures such as cigarette smoke [43–52].

In order to elucidate the cellular and molecular mechanisms underlying the linkage between developmental exposure to cigarette smoke and an increased risk of offspring metabolic disease, we have utilized a well-characterized mouse inhalation exposure model which simulates 'active' smoke exposure spanning both fetal and early neonatal developmental periods (GD1-PD21) and examined the impact of such exposure on the proteome of liver, kidney and hippocampus at varied times during the offspring's lifespan. The present report details the impact of developmental cigarette smoke exposure on hepatic proteome profiles of offspring at 6 months of age – 5 months past cessation of their exposure – from littermates of pups from the same litters utilized in our prior study of developmental (GD1-PD21) cigarette smoke exposure. Parallel studies from our laboratory concerning the impact of such developmental CSE on liver, kidney and hippocampus proteome profiles at weaning (PD21) [53–55] and at adulthood [56, 57] documented an impact of exposure on tissue

metabolic activity. We previously reported that weanling (PD21) offspring who were developmentally exposed to cigarette smoke exhibited hepatic oxidative stress, impaired gluconeogenesis, altered lipid metabolism, impaired small molecule and amino acid metabolism, and impaired cellular morphology networks [54]. In the present report, we document sustained deficits in offspring growth, suppressed serum blood glucose levels, as well as disruption of basal gluconeogenesis in the fed state demonstrating a *continued impact of developmental CSE* on metabolic pathway function in adult offspring aged 6 months well past the cessation of exposure.

## 2. MATERIALS AND METHODS

### 2.1. Animal Exposures

Adult C57BL/6J mice were purchased from Jackson Labs (Bar Harbor, ME). Animals were housed and maintained in the University of Louisville Research Resources Center, an Association for Assessment and Accreditation of Laboratory Animal Care accredited facility. Animals were maintained in a controlled temperature/humidity environment with a 12 hour light/dark cycle and free access to Purina LabDiet 5015 and water throughout the duration of the experiment (both prenatal and postnatal exposure periods as well as following cessation of exposure to cigarette smoke. Female mice were age-matched at the outset of the study and timed pregnancies were obtained by overnight mating of a single mature male with two nulliparous females. The presence of a vaginal plug was considered evidence of mating and the time designated as gestational day 1 (GD 1). Pregnant mice were weighed and randomly assigned to either the Sham exposure (Sham, n=9) or Cigarette Smoke Exposure (CSE; n=9) groups. Animals were exposed from GD1, throughout the entirety of gestation; following parturition maternal animals were exposed with offspring until postnatal day 21 (PD21).

Animals were exposed to ambient air or cigarette smoke for 6 hours per day, 7 days per week, from GD1-PD21. A Teague TE-10C whole body smoke inhalation exposure system (Teague Enterprises; Davis, CA)[58] was utilized to generate and deliver mixed mainstream/sidestream cigarette smoke at the rate of 40 cigarettes smoked per 6 hour period. Cigarette smoke was generated from Philip Morris Marlboro Red brand cigarettes<sup>TM</sup> (Philip Morris; Richmond, VA; 15mg of tar/cigarette; 1.1mg nicotine/cigarette; additives), selected since it represents the most popular brand of cigarettes consumed among 18–25 year olds - the age group containing the majority of maternal smokers [59–62]. Cigarettes were smoked using the standard Federal Trade Commission method: a two second, 35 cm<sup>3</sup> puff, once a minute for a total of 9 min [58]. For the duration of the exposure period, the dam/litter combinations were individually housed in the cages utilized for exposures. For quality control purposes, dual exposure chambers (one receiving cigarette smoke and one receiving ambient air) were characterized twice during each daily exposure session for: total suspended particulates (TSP), temperature, carbon monoxide levels, and humidity (Table 1) [54].

Tail blood was collected from representative animals immediately following the 6 hour exposure session at various time points throughout the 6 week exposure regimen for determination of plasma cotinine levels. Cotinine, the principal metabolite of nicotine, is a well-documented marker of ‘active’ tobacco smoking and passive/environmental tobacco

smoke exposure [63–66]. Cotinine levels were assayed by electrospray tandem mass spectrometry (ESI-MS/MS) utilizing a direct inject platform (Nanomate) coupled to a 7T LTQ-FT-ICR-MS.

Following discontinuation of cigarette smoke exposure on PD21, offspring were maintained at normal temperature and humidity without exposure to any agent. At 6 months of age, following behavioral and cognitive assessments [67], offspring were euthanized by asphyxiation with carbon dioxide followed by thoracotomy and cardiac puncture. Tissues were harvested and stored at  $-80^{\circ}\text{C}$  until analysis. Proteome profiling of the liver and other tissues was conducted on identically exposed littermates at the time of weaning (PD21) [53–55]. The current report of liver proteome profiles from 6 month old animals that were previously developmental exposed to cigarette smoke serves as the lead manuscript for the coordinated three part evaluation of tissue specific proteome profiling studies, and includes the companion studies on kidney and hippocampus proteome profiles [56, 57].

**2.2.1. 2D-SDS-PAGE**—The liver samples (Sham  $n=4$ ; CSE  $n=6$ ) were homogenized in sample preparation buffer [7M urea, 2M thiourea, 40mM dithiothreitol (DTT)]. Protein concentration for each of the samples was determined using the Bradford Assay [68]. Four hundred micrograms of protein in rehydration buffer (8M urea, 2% CHAPS, 2  $\mu\text{l}$  IPG buffer pH 3–10, 2.5 mg/ml DTT, 0.002% bromophenol blue) was applied to IPGphor Drystrips (Nonlinear, 3–11, 180 mm  $\times$  3 mm  $\times$  0.5 mm, GE Healthcare, Piscataway, NJ). First dimension separation by isoelectric focusing at 22,000 Volt hours (Vhrs) was performed with a hold at 100 Volts until further processing. The IEF strips were stored at  $-80^{\circ}\text{C}$  for 1 hr followed by: 1) equilibration for 60 minutes in reducing buffer (6M urea, 75 mM Tris-HCl pH 8.8, 29.3% glycerol, 2% SDS, 0.002% bromophenol blue with 3.5 mg/ml DTT) and 2) equilibration in alkylating buffer (same buffer with 45 mg/ml iodoacetamide instead of DTT) for an additional 30 minutes. Second dimension SDS-PAGE separation (25cm  $\times$  20.5cm 15% polyacrylamide gels) was performed overnight (18 hrs; 100V). Protein spots were visualized by Colloidal Coomassie Blue G-250.

**2.2.2. Image Analysis**—Gels were scanned using an Epson Expression 10000 XL scanner with transparency attachment. Densitometric analysis of gel images was performed with Progenesis SameSpots software (Nonlinear Dynamics; New Castle-on-Tyne, UK). Protein spots were detected automatically and manually adjusted where necessary for accuracy. For each protein spot, the intensity was measured, background was subtracted, and individual spot density was normalized by total pixel density of each gel. Spots with average normalized pixel depth of  $\geq 1000$  relative abundance units and non-normalized areas with pixel depth below 100 were removed as noise. The averaged normalized spot abundance was compared between groups to determine fold differences in abundance.

**2.2.3. Mass Spectrometry Based Protein Identification**—Protein spots were excised and destained with 50% ethanol in 50mM ammonium bicarbonate for a minimum of 5 washes. Excised gel spots were then dehydrated in acetonitrile (ACN), dried, and rehydrated with 10 ng/ $\mu\text{l}$  trypsin and 40mM ammonium bicarbonate. Proteins were digested at room temperature for approximately 18 hrs. Peptides were eluted in acidified acetonitrile and stored at  $-20^{\circ}\text{C}$  until analysis. The mass to charge ratio of peptides was determined by direct

inject LTQ/FT-ICR-MS/MS (or HPLC interface on occasion) with collision induced dissociation for structural feature identification. Peptide identification was performed with the Mascot (Matrix Sciences v 2.2.2) search algorithm utilizing the NCBI nr (with decoy) database (updated June 1, 2010). Search parameters included: mammalian class, 2 missed cleavages, carbamidomethyl C variable modification, enzyme trypsin/P, and an allowed peptide charge of 1+, 2+, or 3+. Positive protein identification required a total MOWSE absolute probability entire protein score of  $\geq 100$  composed of a minimum of two peptides with individual scores MOWSE absolute probability scores  $\geq 50$  [53–55].

**2.2.4. Statistical Analysis**—Analysis of Variance (ANOVA, two way,  $p < 0.05$  as significant) and a series of Partial Least Squares-Discriminant Analysis (PLS-DA) models were utilized to determine the protein spots which differed in intensity and described the differences between the groups. Multiple PLS-DA models were constructed utilizing Variable Importance in Projection (VIP)-ranked protein spots of interest with recursive feature elimination identifying protein spots with  $VIP \geq 1.75$  as important in defining the separation between groups [69]. Sequential removal of top ranked protein spots was performed until the variance between groups was eliminated. All protein spots included in further analysis were significantly different between groups based on ANOVA ( $p < 0.05$ ).

### 2.3. Ingenuity® Pathway Analysis

Ingenuity® Pathway Analysis was used to determine families of proteins and metabolic pathways of the proteins that were identified (Ingenuity Systems, 2010). Once the proteins were identified, the GI numbers of the proteins were entered into the IPA algorithm. Networks of interactions between the proteins and their respective genes were generated by the program. Networks of interactions between the proteins and biological pathways were generated with sub-categorization by increased or decreased abundance. For the canonical pathways analysis, the following settings were employed: Benjamin-Hochberg p-value greater than or equal to 1.5, a threshold of 0.5 z-score, and a Benjamin-Hochberg Multiple Testing Correction p-value utilized for scoring. In the associated figure (Figure 5), solid lines indicated a direct interaction while dotted lines indicate an indirect interaction. Geometric shapes identify classes of proteins: phosphatases (triangle), kinases (inverted triangle), enzymes (vertical diamond), transcription regulators (horizontal ellipse), transporters (trapezoid), and other important molecules (circles).

### 2.4. Serum Glucose

Serum glucose levels in offspring in the fed-state were analyzed by a handheld glucometer (OneTouch Ultra, LifeScan Inc.) in representative offspring from 5 litters per group.

### 2.5. Glutathione (GSH and GSSG) Assay

The ratio of reduced/oxidized glutathione (GSH/GSSG) was utilized as an indicator of oxidative stress. To measure total glutathione (GSH + 2GSSG), GSH standards (or sample homogenate) was combined with 50mM phosphate buffer (pH=7.2) containing 2mM EDTA with the addition of 2 $\mu$ Units of glutathione reductase, and incubated in the presence of DNTB for 30 minutes in the dark followed by spectral acquisition at 405nm [70, 71]. A separate GSSG standard curve was constructed for GSSG measurement with the

modification of a pre-incubation of samples or standards with 100mM 2-vinylpyridine for 60 minutes followed by addition of glutathione reductase and subsequent incubation in the presence of DNTB as described for total glutathione measurements. All glutathione measurements were normalized to total protein as measured by the Bradford assay [68]. The amount of free GSH was calculated from the total glutathione minus the GSSG levels in each sample. The ratio of GSH/GSSG was then calculated.

## 2.6. Glutathione-S-Transferase (GST) and Glutathione Reductase (GR) Assay

Liver GST activity was measured as an indicator of detoxification activity [72]. The total GST activity of the liver was measured using the enzyme driven conjugation of 1-chloro-2,4-dinitrobenzene (CDNB) to reduced glutathione (absorbance read at 340 nm each minute for 15 minutes; Cayman Chemical Company). The absorbance per minute was divided by amount of protein (mg) to determine the specific activity for each sample [73]. Glutathione reductase activity was measured spectrophotometrically by mixing an aliquot of tissue homogenate with GSSG and measuring the GST enzyme driven conjugation to 1-chloro-2,4-dinitrobenzene (CDNB) to reduced glutathione (absorbance read at 340 nm each minute for 15 minutes; Cayman Chemical Company) [74].

## 2.7 Western Blot

Liver protein homogenates from the 2D-SDS-PAGE preparations were mixed 1:1 with Laemmli buffer (0.25M Tris pH 6.8, glycerol, 10% SDS, bromophenol blue trace) then heated at 70° C for 10 minutes. Twenty-five µg total protein was separated by 10% PAGE for 2 hours at a 100V in Tris-glycine run buffer (0.025 M Tris Base, 0.192 M glycine, 0.1% SDS) followed by electrophoretic transfer to PVDF membrane at 90V for 1 hour in transfer buffer (0.025M Tris base, 0.192M glycine, 10% ethanol). Following blocking in 4% non-fat dry milk, the blots were incubated overnight at 4°C with primary antibody diluted 1:500 in non-fat dry milk (SIRT1, sc-19857; PEPCK, sc-271029; PGC1α, sc-13067; Santa Cruz Biotechnology, Dallas, TX). After three washes of 15 minutes in cold PBS-Tween, blots were incubated with secondary antibody complexed to horse radish peroxidase (1:1000, Santa Cruz Biotechnology, Dallas, TX) diluted in non-fat dry milk at room temperature for 1 hour. After three washes in cold PBS-Tween, blots were developed with chemiluminescence substrate and visualized with an ImageQuant LAS 4000 (G.E. Healthcare Life Sciences, Pittsburgh, PA). Following visualization, blots were washed three times with PBS-Tween, incubated in 2M glycine for 30 minutes followed by another three washes in PBS-Tween and subsequent incubation with β-actin primary antibody (1:1000 dilution; sc-81173, Santa Cruz Biotechnology, Dallas TX) and secondary antibody and visualization as described above [75].

## 3. RESULTS

### 3.1. Exposure Conditions and Offspring Weights

Mean CO and TSP levels in the cigarette smoke exposure chamber were  $138 \pm 19.8$  ppm and  $25.4 \pm 6.5$  mg/m<sup>3</sup>, respectively with measures in the Sham group found to be less than the limit of detection for each assay. Cotinine, a metabolite of nicotine, was used as an indicator of cigarette smoke exposure. Cotinine levels were greater than 50ng/mL in the CSE group

indicating an ‘active’ exposure model with Sham exposure group cotinine levels below the detection limit of 4 ng/mL (Table 1). Low birth weight was evident in the CSE offspring (~15% decrease relative to Sham) [54] with persistence of decrements in weight throughout weaning and into maturity (maintenance on Purina 5015 diet) [67]. Six month old offspring from the CSE group remained reduced in weight at the time of cognitive and behavioral assessment [67] and at the time of tissue collections for the current study (data not shown).

### 3.2. Liver Proteome Profiles

As shown in Figure 1, 2D-SDS-PAGE gels of liver proteins from Sham and CSE mice were similar at 6 months of age (5 months since cessation of exposure to cigarette smoke). The proteins on the gels spanned an isoelectric focusing range of 3 to 10, with the acidic proteins on the left and the basic proteins on the right of the gel image and descending molecular weights ranging from ~80 kDa to ~11 kDa. The predominant variance between groups was in the intensity of protein spot abundances rather than the appearance/disappearance of protein spots. The average  $\pm$  standard deviation of the total spot density of the Sham group equaled 4,814,147,401  $\pm$  277,980,203 while the CSE group equaled 4,827,210,729  $\pm$  282,064,550.

### 3.3. Partial Least Squares- Discriminant Analysis

Iterative PLS-DA models were generated encompassing spot abundances of all proteins not determined to be noise; sequential removal of top VIP ranked protein spots contributing to the separation of groups followed by re-plotting of group separation until the loss of separation of groups resulted in identification of a set of features that were essential to describing the differences between groups. Sixty protein spots (VIP 1.75,  $p < 0.05$ ) were found to contribute to the separation in proteome profiles between the Sham and CSE groups. When abundances of all protein spots were included (noise excluded), the first latent factor of the PLS-DA model accounted for 87% of the variance between the Sham and CSE groups and the second latent factor accounted for an additional 12% of the variance. As shown in Figure 2, the proteome profiles of the Sham and CSE groups are distinct. The separation between the groups is depicted by graphing latent factors 1 and 2.

### 3.4. Proteins Impacted by CSE

Forty-two proteins of interest were identified by proteolytic digestion and ESI-MS/MS. These proteins represented all spots of sufficient intensity for clarity of boundaries and all spots that included p-values within 0.10. Identification of the remaining protein spots was not attempted although they did not represent background noise. Of the 42 protein spots whose identification was attempted, 36 were identified unambiguously (see Table 2). Proteins that represent the predominant contribution to the spot intensity, as determined by a minimum of 200% of the MOWSE score of the next ranked protein identified, are listed. Proteins with altered abundance in liver of CSE mice when compared to that of Sham-exposed mice were grouped by membership in metabolic networks via IPA analysis. As shown in Figure 3, proteins with altered abundance in the liver of 6 month old mice previously developmentally exposed to cigarette smoke belonged to the Sucrose Degradation, NRF2-mediated Oxidative Stress Response, Glycolysis, Gluconeogenesis, Methionine Degradation, Methylglyoxal Degradation, and Aldosterone signaling pathways.

**3.4.1. Carbohydrate Metabolism Impacted by CSE**—The abundance of two proteins belonging to the *Sucrose Degradation* pathway (total of nine protein members for a 22% pathway coverage) was altered in the liver of 6 month old offspring who were developmentally exposed to cigarette smoke; dihydroxyacetone kinase 2 (**Spot 7**; decreased 37%) and ketohexokinase (**Spot 19**; increased 39%). The conversion of dihydroxyacetone to the phosphorylated form utilizes ATP as a cofactor, while the conversion of D-fructose to D-fructose-1-phosphate generates AMP. The opposing impact may signal a cellular prioritization of ATP utilization for energetic processes. Enolase 1 (**Spot 22**; increased 47%) is a member of both the *Glycolysis/Gluconeogenesis* pathways while fructose-1, 6-bisphosphatase 1 (**Spot 20**; increased 43%) is a key regulatory step of the gluconeogenesis pathway; both of these enzymes were increased in the liver of adult offspring aged six months who were developmentally exposed to cigarette smoke. The *Gluconeogenesis* pathway has a total of 9 direct and 16 support members (2/9 and 22% or 2/25 and 8% coverage respectively). As shown in Figure 4, the hepatic carbohydrate metabolism pathway was impacted in offspring aged 6 months who had undergone developmental exposure to cigarette smoke, with insulin signaling likely impacted. Serum glucose levels in these same CSE offspring were decreased (~15%; Figure 5). As validation of the purported impact of developmental CSE on hepatic gluconeogenesis at a time 5 months past cessation of exposure, we measured the expression of three metabolic regulatory proteins that influence gluconeogenesis protein expression (Figure 6A–6C). SIRT1 expression was inversely correlated to PEPCK in the current study, similar to the established literature, though the current study did not directly measure SIRT1 enzymatic activity [76]. PGC1 $\alpha$ , which enhances gluconeogenic gene expression and is a known target of SIRT1 deacetylase activity, exhibited increased expression in the current study.

**3.4.2. NRF2-mediated Oxidative Stress Response Impacted by CSE**—The abundance of four proteins belonging to the *NRF2-mediated Oxidative Stress Response* pathway (180 members of pathway with total coverage of 2.2%) was altered in the liver of 6 month old adult offspring by developmental CSE:  $\gamma$ -actin (**Spot 16**; increased 34%), glutathione S-transferase  $\mu$  (**Spot 33**; increased 15%; GST), peroxiredoxin 1 (**Spot 34**; increased 37%; PXR1), and transitional endoplasmic reticulum ATPase (**Spot 3**; decreased 21%; ER ATPase). The pool of glutathione (reduced, GSH; oxidized, GSSG; and total, GSH+2GSSG; Figure 7) was decreased by 14% in the liver of adult offspring who were developmentally exposed to cigarette smoke indicating possible suppression of glutathione synthesis though a key marker of oxidative stress, the GSH/GSSG ratio remained unaltered and no impact on GR or GST activity was noted (data not shown).

**3.4.3. Amino Acid Degradation Impacted by CSE**—The abundance of betaine-homocysteine S-methyltransferase (**Spot 10**; increased 47%) and adenosylhomocysteinase (**Spot 14**; increased 80%), two enzymes involved in methionine degradation (2/32 members, 6.2 % pathway coverage), was increased within the liver of 6 month old adult offspring following developmental CSE. The abundance of 3-hydroxyisobutyrate dehydrogenase (**Spot 27**; increased 39%; valine degradation), isovaleryl-CoA dehydrogenase (**Spot 21**; increased 86%; leucine degradation); and arginase (**Spot 23**; increased 51%; arginine metabolism to urea) also was increased in the liver of the adult offspring who were



developmentally exposed to cigarette smoke. These five enzymes participate in amino acid metabolism, including the modulation of branched chain amino acid catabolism in support of gluconeogenesis.

**3.4.4. Aldosterone Signaling Impacted by CSE**—The abundance of adenosylhomocysteinase (**Spot 14**; increased 80%), HSP 90 (**Spot 17**; decreased 32%) and HSP 60 (**Spot 5**; decreased 35%) were altered in the liver of adult offspring aged 6 months who had undergone developmental exposure to cigarette smoke. Two members of this pathway are involved in the chaperoning of other proteins during cellular stress. Similarly, three proteins with increased abundance from the NRF-2 mediated oxidative stress response pathway were identified as cellular stress response proteins (GST, PXR1, and ER ATPase) with peroxiredoxin 6 (**Spot 32**, increased 30%) also acting as an antioxidant and cellular stress response protein. Coupled with the state of oxidative stress, heightened hepatic stress likely exists in the 6 month old adult offspring, who were developmentally exposed to cigarette smoke, that would lead to an impaired response to a secondary stressor.

**3.4.5. Cytoskeletal Structural Proteins Impacted by CSE**—The abundance of radixin (**Spot 2**, increased 95%), actin 2 (**Spot 16**, increased 34%), and regulator of microtubule dynamics protein 1 (**Spot 31**, increased 120%) was increased in the liver of 6 month old adult offspring developmentally exposed to cigarette smoke. Radixin 1 regulates head-to-tail association and plasma membrane binding of actin filaments while regulator of microtubule dynamics protein 1 modulates microtubule assembly. An increase in the levels of these proteins indicates an increased cellular requirement for cytoskeletal stabilization protein expression likely in response to increased oxidative stress.

**3.4.6. Lipid Metabolism Proteins Impacted by CSE**—The abundance of delta(3,5)-delta(2,4)-dienoyl-CoA isomerase (**Spot 29**; increased 55%; fatty acid isomerization) and glycerol-3-phosphate dehydrogenase (**Spot 25**; increased 69%; links carbohydrate and lipid metabolism) was increased in the liver of adult offspring aged 6 months who had undergone developmental exposure to cigarette smoke, possibly signaling an increase in fatty acid oxidation and mitochondrial activity. A trend toward increased serum triglycerides was found ( $p=0.09$ ; data not shown) in these same CSE offspring.

## 4. DISCUSSION

The mouse model of developmental CSE (GD1-PD21) that was employed in the present study exhibited low birth weight and sustained suppression of weight at both weaning (PD21; time of cessation of exposure) and at PD180 - the time of tissue collection for the present study [54]. Serum cotinine levels in excess of 50 ng/mL – as utilized in the current study – are typically classified as an ‘active’ smoking exposure paradigm [77]. The sustained decrements in weight of 6 month old adult offspring who were developmentally exposed to ‘active’ cigarette smoke are a feature of the current study and the parallel studies on the hippocampus and kidney proteomes of these animals [56, 57]. As such, the exposure system models low birth weight outcomes in infants whose mothers smoked throughout gestation [54, 67, 78, 79] and mirrors the persistence of weight decrements seen in such exposed individuals through childhood and into adulthood [80–84]. The impact of developmental

cigarette smoke exposure likely reflects systemic toxicity that is coordinately manifested within individual tissues as reflected within the proteomics studies reported within this model system and in conjunction with the prior report describing behavioral alterations within these same offspring [53–57, 67].

In a study examining the impact of gestational ‘passive’ cigarette smoke exposure on mouse fetal liver, genotoxic damage (including DNA adduct formation and oxidative nucleotide damage) was observed, along with transcriptomic alterations in xenobiotic metabolic pathways; stress response proteins; protein repair mediators, protein removal and folding effectors; cell cycle regulators; growth factors; and cytoskeletal proteins [38]. We previously reported that juvenile offspring (i.e. at PD21 at weaning) who were developmentally exposed to cigarette smoke in an identical manner to the current study, exhibited alterations in proteomic abundances including antioxidant proteins (mixed impact), glucose metabolic enzymes (suppressed gluconeogenesis), and protein members of metabolic networks including lipid metabolism (mixed impact), small molecule and amino acid metabolism (mixed impact), and cellular morphology networks (inhibited) [54]. In the present report, we document a *continued impact of developmental CSE* on several of these same metabolic pathways in 6 month old adult offspring, 5 months post-cessation of exposure.

The carbohydrate metabolic pathway was impacted in the liver of 6 month old offspring who previously were developmentally exposed to cigarette smoke with insulin signaling likely impacted. In the current study, proteins enolase 1 (gluconeogenesis/glycolysis pathway) and fructose-1, 6-bisphosphatase (gluconeogenesis only) were increased which points to a dysregulation of glucose metabolic activity within the fed-state. A mixed impact on dihydroxyacetone kinase 2 and ketohexokinase was also observed. The conversion of dihydroxyacetone to the phosphorylated form utilizes ATP as a cofactor while the conversion of D-fructose to D-fructose-1-phosphate generates AMP. This opposing impact may signal a cellular prioritization of ATP utilization for energetic processes. Serum glucose levels in these same CSE offspring were decreased and point to the limited systemic availability of energetic substrates. SIRT1 expression, an NAD<sup>+</sup>-dependent protein deacetylase, coordinately regulates hepatic gluconeogenesis through the post-translational modification of PGC1 $\alpha$  [85] and leads to the deacetylation and degradation of the cAMP response element binding protein (CREB) regulated transcription coactivator 2 (CRTC2) with attenuation of gluconeogenesis [86]. In the current study, we report a suppression of SIRT1 protein expression which was inversely associated with PEPCK and PGC1 $\alpha$  protein expression. We believe this is the first report of an impaired “switch” from gluconeogenesis to glycolysis in the fed state in 6 month old adult animals that were previously developmentally exposed to cigarette smoke. We propose that the failure to equivalently limit hepatic gluconeogenesis in the fed state acts as a survival mechanism to provide additional systemic glucose and may indicate a failure to adequately respond to insulin signaling with the attendant implication that mitochondrial insufficiency and leakage may lead to diet-associated metabolic syndrome.

Systemic oxidative stress is a characteristic feature of ‘active’ CSE in similar animal model systems. At weaning (PD21) in our model system, hepatic oxidative stress was evident at the time of cessation of developmental CSE [54]. In the present study we report a sustained

decrement in the availability of glutathione pools (total, free, and oxidized) though the ratio of GSH/GSSG remained unchanged in 6 month old adult offspring who were exposed throughout developmental to cigarette smoke. This lack of reducing equivalents, coupled with the increase in antioxidant enzymes, indicates a systemic stress response.

DNA methylation is dependent on one-carbon metabolism and homocysteine availability. A recent report demonstrated that maternal smoking during pregnancy resulted in sex-specific impairment of one-carbon metabolism in human fetal liver [87]. We report that betaine-homocysteine S-methyltransferase and adenosylhomocysteinase, two enzymes involved in methionine degradation and specifically within the one-carbon metabolic pathway, were increased in abundance in the liver of 6 month old adult offspring who had undergone prior developmental cigarette smoke exposure. These alterations were coupled to a more generalized increase in the abundance of amino acid catabolic enzymes and indicate increased requirements for DNA methylation substrates as well as gluconeogenic precursors.

In summary, 'active' CSE from GD1-PD21 results in growth retardation that persists into adulthood well past the cessation of offspring exposure. Liver metabolic networks including glucose metabolism that were suppressed in PD21 weanling animals at the time of cessation of exposure continued to be impacted in adults, though gluconeogenic activity in the fed state appears to be elevated, with an accompanying suppression of fed-state serum glucose availability. We propose that a continued suppression of energetic precursor availability not only impacts hepatic metabolic pathways but influences systemic metabolic function. The accompanying hepatic oxidative stress that is present both in PD21 animals at the time of cessation of exposure as well as in 6 month old adult animals likely influences both cellular/tissue structure and function. The developmental basis of adult disease states that an adverse environment during development yields a propensity to diet-induced obesity, metabolic syndrome, diabetes, and a host of associated deleterious health outcomes. We propose that developmental exposure to 'active' maternal cigarette smoking falls within this paradigm. The observed hepatic metabolic dysregulation noted in exposed offspring may influence the development of metabolic syndrome at maturity within a high fat diet challenge paradigm. In future studies, we will address the impact of developmental 'passive' and 'active' cigarette smoke exposure on offspring sex-specific hepatic function within the context of diet-induced metabolic syndrome.

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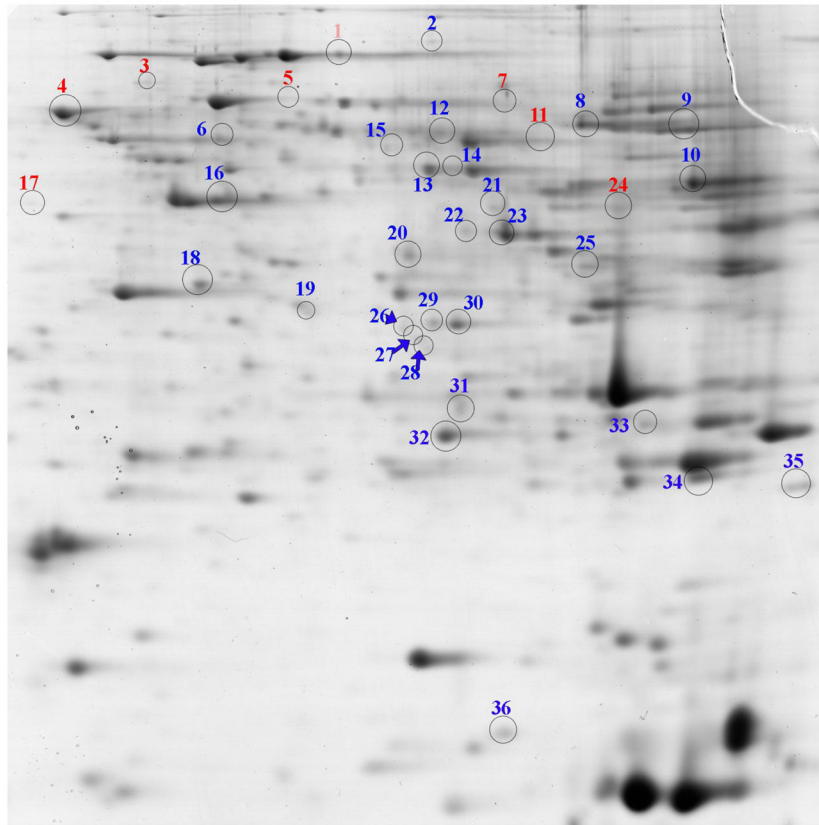
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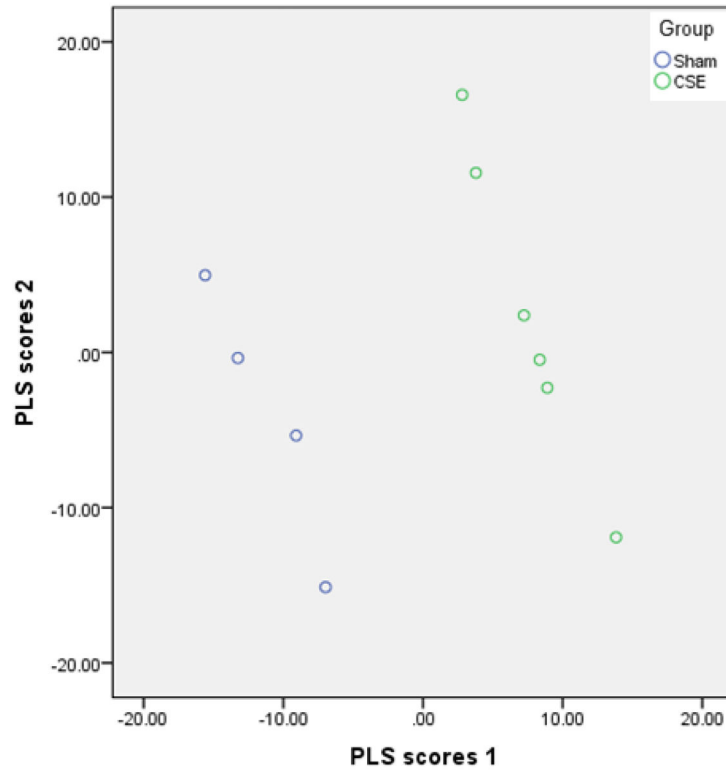
**Highlights**

- Developmental exposure to cigarette smoke results in persistent alterations in hepatic proteome.
- Sustained impairment of carbohydrate metabolism in 6 month old CSE offspring
- Weight gain may reflect impaired carbohydrate metabolism.

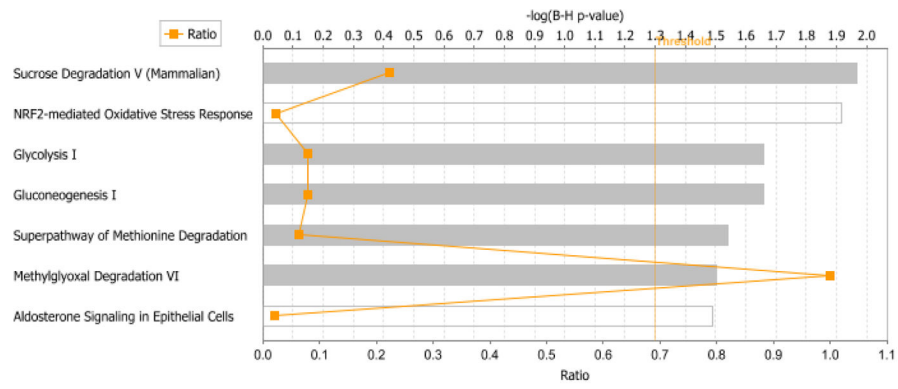


**Figure 1. Liver proteome profiles of 6 month old offspring previously developmentally exposed to cigarette smoke**

The proteins with altered abundance that contributed to the separation of the groups within the PLS-DA model and possessed the highest VIP values ( 1.75) are numbered. Numbers in blue represent increased abundance and the numbers in red represent decreased abundance. The gel image is highly contrasted to enable visualization of low abundant protein spots. Refer to Table 2 for protein identifications.



**Figure 2. The PLS-DA model effectively describes the differences between the liver proteome profiles of Sham and developmental CSE offspring at 6 months of age**  
Plotting the first two vectors within the PLS-DA model visualizes the variance in liver proteome profiles between the Sham and CSE groups. Each circle represents a single sample subjected to 2D-SDS-PAGE.



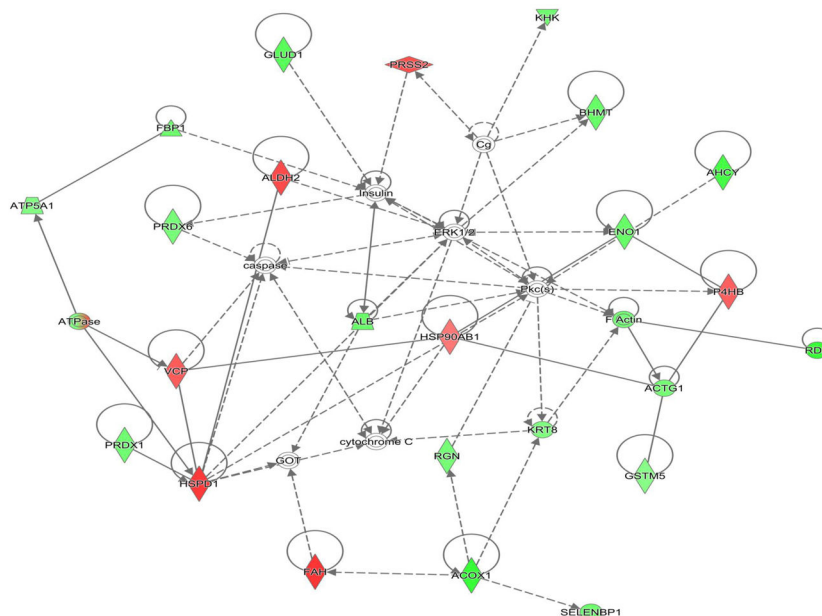
**Figure 3. The top seven ranked protein interaction networks and pathways impacted within the liver of 6 month old offspring who were previously developmentally exposed to cigarette smoke**  
The distance from the threshold value (vertical orange line) depicts the intensity of change between Sham exposure and CSE groups.

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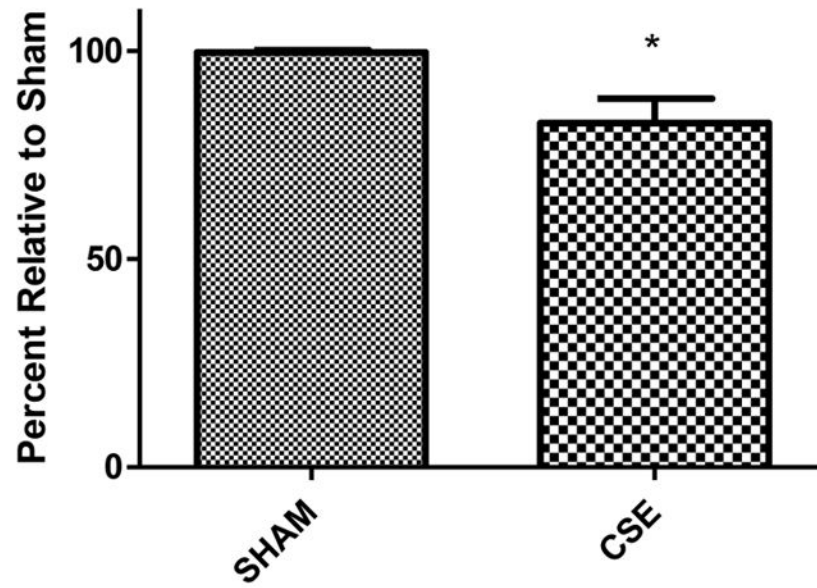
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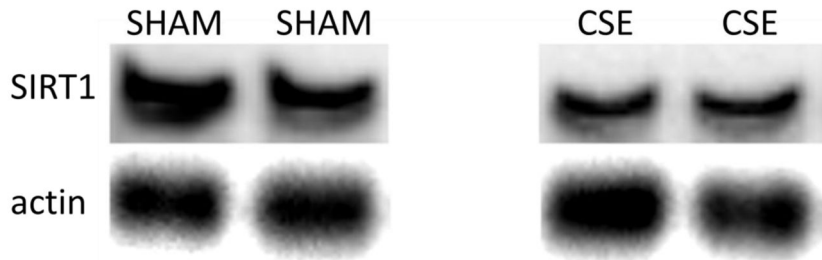
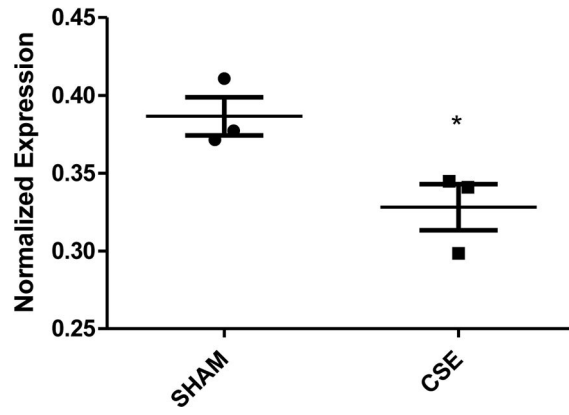
**Figure 4. The hepatic carbohydrate metabolism pathway in 6 month old offspring is impacted by previous developmental CSE**

As shown below, insulin signaling is a central node that regulates an abundance of metabolic proteins found to be altered in the liver of offspring developmentally exposed to cigarette smoke. In the associated figure, solid lines indicated a direct interaction while dotted lines indicate an indirect interaction. Geometric shapes identify classes of proteins: phosphatases (triangle), kinases (inverted triangle), enzymes (vertical diamond), transcription regulators (horizontal ellipse), transporters (trapezoid), and other important molecules (circles).



**Figure 5. Serum glucose levels in 6 month old offspring that were sham exposed or exposed to cigarette smoke from GD1 through PD21**  
Fed state serum glucose levels, collected at the time of euthanization, are suppressed by 17% in CSE offspring at 6 months of age (\* $p < 0.0001$ ).

Figure 6A



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Figure 6B

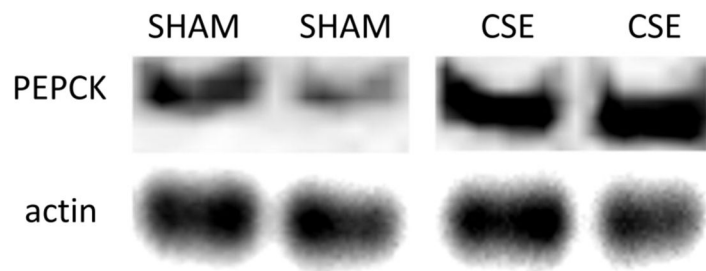
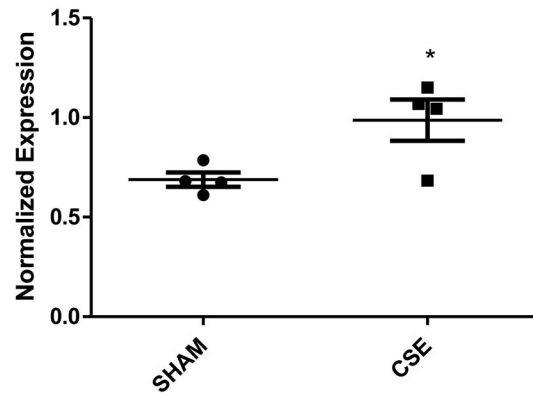
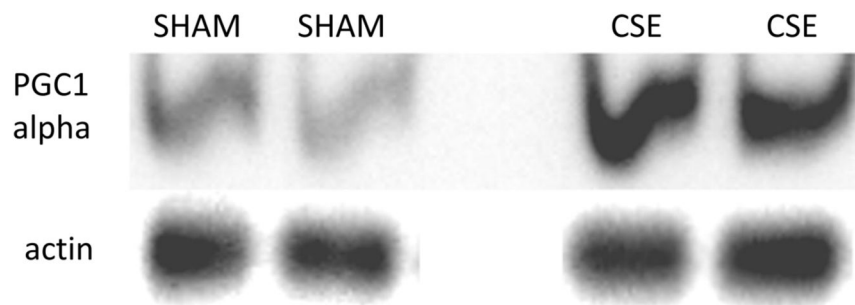
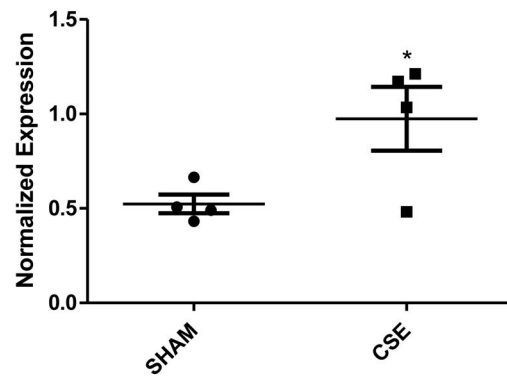


Figure 6C



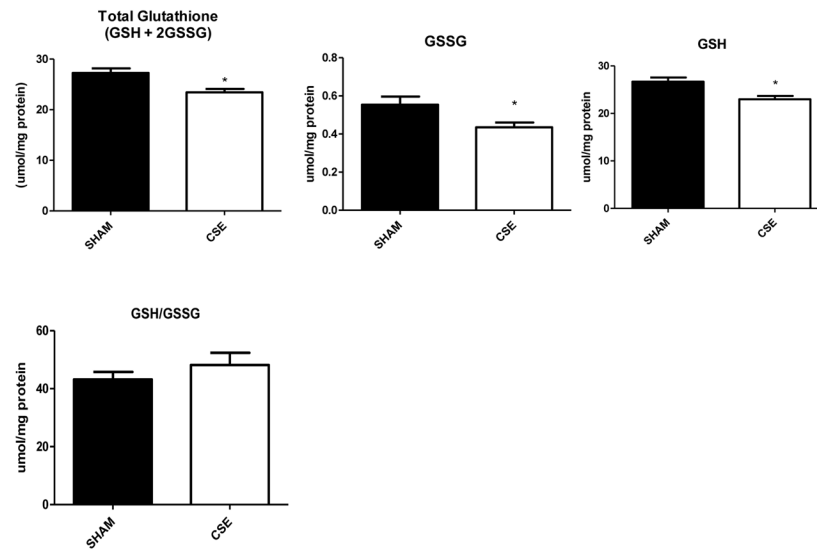


**Figure 6.**

**Figure 6A: Western blot analysis of SIRT1 expression in whole liver homogenates from 6 month old offspring that were sham exposed or exposed to cigarette smoke from GD1 through PD21.** Liver homogenates from CSE offspring at age 6 months exhibited a decrease of approximately 10% in expression of the metabolic regulatory protein SIRT1 (\* $p < 0.05$ ;  $n = 3$  per group).

**Figure 6B: Western blot analysis of PEPCK expression in whole liver homogenates from 6 month old offspring that were sham exposed or exposed to cigarette smoke from GD1 through PD21.** Liver homogenates from CSE offspring at age 6 months exhibited an increase of approximately 44% in PEPCK expression in the fed state (\* $p < 0.05$ ;  $n = 4$  per group).

**Figure 6C: Western blot analysis of PGC1 $\alpha$  expression in whole liver homogenates from 6 month old offspring exposed to cigarette smoke from GD1 through PD21.** Liver homogenates from CSE offspring at age 6 months exhibited an increase of approximately 86% in PGC1 $\alpha$  expression in the fed state (\* $p < 0.05$ ;  $n = 4$  per group).



**Figure 7. Glutathione levels in liver of 6 month old offspring that were sham exposed or exposed to cigarette smoke from GD1 through PD21**

Developmental CSE reduces hepatic glutathione levels at maturity. Hepatic total glutathione (GSH+2GSSG), oxidized glutathione (GSSG), and reduced glutathione (GSH) were reduced at maturity in offspring developmentally exposed to cigarette smoke (n=7 per group, \*p<0.05) without an impact on the overall reduced/oxidized glutathione ratio (GSH/GSSG).

**Table 1**  
**Animal exposure conditions and outcomes**

Mean Carbon Monoxide and Total Suspended Particulates (TSP) were calculated from measures taken twice daily in the inhalation exposure chambers from GD1-PD21. Plasma cotinine levels were determined by tail vein blood sampling of the dam and offspring within 1 hour of cessation of exposure on PD21 (\*p < 0.05).

Outcome/Condition	SHAM	CSE
Carbon Monoxide (ppm)	0	138 ± 19.8*
TSP (mg/m <sup>3</sup> )	0	25.4 ± 6.5*
Dam Cotinine (ng/mL)	<4	89.7 ± 37.3*
Pup Cotinine (ng/mL)	<4	244.3 ± 132.4*

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Table 2

**Protein spots with altered abundances that were ranked of greatest import within the PLS-DA model, VIP values ( 1.75; p<0.05)**

Numbers in red represent decreased abundance and the numbers in blue represent increased abundance of proteins in 6 month old offspring previously developmentally exposed to cigarette smoke (GDI-PD21), as compared to the Sham group. Corresponds to Figure 1.

Spot Number	GI Number	Abbreviation <sup>1</sup>	Protein Identification	VIP	Percent Change
1	24691	PRSS2	Antic trypsin-1	1.92	-26
3	1174637	VCP	Transitional endoplasmic reticulum ATPase	2.00	-21
4	341941239	P4HB	Protein disulfide-isomerase	2.25	-19
5	51702252	HSPD1	60 kDa heat shock protein, mitochondrial	2.13	-35
7	225913		Bifunctional ATP-dependent dihydroxyacetone kinase/FAD-AMP lyase (cyclizing)	1.78	-37
11	11669	ALDH2	Aldehyde dehydrogenase, mitochondrial	1.87	-29
17	15516	HSP90AB1	Heat shock protein HSP 90-beta	2.02	-32
24	14085	FAH	Fumarylacetoacetase	1.92	-36
2	341942170	RDX	Radixin	2.07	95
6	16691	KRT8	Keratin, type II cytoskeletal 8	1.77	
8	14661	GLUDI	Glutamate dehydrogenase 1, mitochondrial	1.98	61
9	Q03265	ATP5A1	ATP synthase subunit alpha, mitochondrial	1.80	21
10	12116	BHMT	Betaine-homocysteine S-methyltransferase 1	1.89	47
12	20342	SELENBP1	Selenium-binding protein 2	1.97	80
13	11657	ALB	Serum albumin	1.96	46
14	29443	AHCY	Adenosylhomocysteinase	2.18	80
15	52815		Probable D-lactate dehydrogenase, mitochondrial	1.88	53
16	11465	F-actin	Actin, cytoplasmic 2	1.84	34
18	19733	RGN	Regucalcin	1.89	34
19	25659	KHK	Ketohexokinase	1.99	39
20	14121	FBP1	Fructose-1,6-bisphosphatase 1	2.20	43
21	56357		Isovaleryl-CoA dehydrogenase, mitochondrial	1.93	86
22	1363776	ENO1	Alpha-enolase	1.78	47
23	11846		Arginase-1	1.82	51
25	14555		Glycerol-3-phosphate dehydrogenase (NAD <sup>+</sup> ), cytoplasmic	2.01	69

Spot Number	GI Number	Abbreviation <sup>1</sup>	Protein Identification	VIP	Percent Change
26	107766		3-hydroxyanthranilate 3,4-dioxygenase	1.99	41
27	63938		3-hydroxyisobutyrate dehydrogenase, mitochondrial	2.25	39
28			Not identified	1.81	23
29	3122065		Delta(3,5)-Delta(2,4)-dienoyl-CoA isomerase, mitochondrial	1.95	55
30	107766		3-hydroxyanthranilate 3,4-dioxygenase	1.86	42
31	66302		Regulator of microtubule dynamics protein 1	1.85	120
32	3219774	PRDX6	Peroxiredoxin-6	1.98	30
33	14862	GSTM5	Glutathione S-transferase Mu 1	2.27	15
34	18477	PRDX1	Peroxiredoxin-1	2.14	37
35	11430	ACOX1	Peroxisomal acyl-coenzyme A oxidase 1	2.26	95
36	20042		40S ribosomal protein S12	1.75	49

<sup>1</sup> Denotes abbreviation used in Figure 4. VIP (Variable Importance in Projection); GI Number (NCBI protein sequence *GenInfo Identifier*).