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Developmental Programming: Changes in Mediators of Insulin Sensitivity in Prenatal Bisphenol A-treated Female Sheep

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Abstract

Developmental exposure to endocrine disruptor bisphenol A (BPA) is associated with metabolic defects during adulthood. In sheep, prenatal BPA treatment causes insulin resistance (IR) and adipocyte hypertrophy in the female offspring. To determine if changes in insulin sensitivity mediators (increase in inflammation, oxidative stress, and lipotoxicity and/or decrease in adiponectin) and the intracrine steroidal milieu contributes to these metabolic perturbations, metabolic tissues collected from 21-month-old female offspring born to mothers treated with 0, 0.05, 0.5, or 5 mg/kg/day of BPA were studied. Findings showed prenatal BPA in non-monotonic manner (1) increased oxidative stress; (2) induced lipotoxicity in liver and muscle; and (3) increased aromatase and estrogen receptor expression in visceral adipose tissues. These changes are generally associated with the development of peripheral and tissue level IR and may explain the IR status and adipocyte hypertrophy observed in prenatal BPA-treated female sheep.

Keywords

Endocrine disruptor; bisphenol A; insulin resistance; oxidative stress; inflammation; lipotoxicity

INTRODUCTION

While communicable diseases were the major causes of mortality during the 18th and 19th centuries, currently non-communicable diseases (NCD), such as cancer and cardio-metabolic diseases, are the leading cause of death worldwide [1, 2]. One of the causes attributed for the rise in NCD is developmental exposure to man-made environmental

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chemicals resulting from increased industrialization. This is supported by the data from the United States (US) Centers for Disease Control and Prevention national biomonitoring program which determined that almost all Americans have detectable levels of a wide variety of environmental chemicals including endocrine-disrupting chemicals (EDCs) in their bodies [3]. Both Centers for Disease Control and Prevention and the National Health and Nutrition Examination Survey (NHANES) have found that bisphenol A (BPA), one such EDC, is detectable in >90% of the US population [4, 5].

BPA is used in the production of epoxy and polycarbonate resins that are used in the manufacture of common household plastics. The main endocrine disrupting function attributed to BPA is its estrogenic activity [6–8] but other properties including antiandrogen [9], thyroid modifying [10] and metabolic functions [7, 8] are described. Epidemiological surveys in the humans have linked BPA exposure to metabolic defects including insulin resistance (IR), diabetes, obesity and metabolic syndrome [11–13]. Although associations between prenatal exposure to BPA and adverse anthropometric birth outcomes have been documented from human cohort studies [14, 15], studies linking prenatal exposure to BPA to adult onset metabolic disorders are limiting. However, presence of high levels of BPA in human maternal circulation during first trimester have been linked to reduced birth weight [15], a major risk factor for adult onset cardio-metabolic disorders [16]. Studies in various animal models provide direct evidence that developmental exposure to BPA leads to metabolic defects [17–19]. Gestational BPA exposure in sheep, a precocial species like humans [20] and hence of translational value, induces metabolic defects in the female offspring at adult age characterized by peripheral IR with compensatory hyperinsulinemia, adipocyte hypertrophy in visceral adipose tissue (VAT), and elevated markers of oxidative stress in the circulation and VAT [19, 21]. However, the mechanisms that lead to the development of these insulin sensitivity and adipocyte defects are not completely understood.

Various factors are implicated in the development of IR including increase in factors that negatively affect insulin sensitivity such as chronic low-grade inflammation, dyslipidemia, oxidative stress, and lipotoxicity in metabolic tissues [22–24] with or without decrease in factors that promote insulin sensitivity such as antioxidants and adiponectin [25, 26]. Inflammation is characterized by activation of resident macrophages leading to production of proinflammatory cytokines that attract infiltration of additional macrophages setting up a vicious cycle of proinflammatory cytokine production that deactivate members of the insulin signaling thereby interfering with tissue insulin sensitivity [23, 27]. Infiltration of macrophages also increases oxidative stress [28] and promotes lipolysis leading to dyslipidemic state [24], which can cause ectopic accumulation of lipids and lipotoxicity in liver and muscle. Lipids negatively affect glucose metabolism and interfere with insulin action and contribute to development of IR [29]. Similarly, development of oxidative stress can oxidize or nitrosylate proteins involved in insulin signaling, reduce gene expression and damage cellular components leading to reduced insulin sensitivity [30]. On the contrary the decrease in positive mediator of insulin sensitivity such as adiponectin (ADIPOQ), a adipokine produced predominantly by adipose tissue [31], is also strongly associated with metabolic disorders [26]. It is present in the circulation in oligomeric low- (LMW), middle- (MMW), and high-molecular weight (HMW) forms, of which the HMW form is the

metabolically active form [32]. Antioxidants such as glutathione reductase (GSR) and superoxide dismutases (SOD) are cellular defenses against reactive species and promote insulin sensitivity by reducing oxidative stress [25].

Steroids also have a role in regulating adipocyte proliferation, differentiation [33], insulin sensitivity and inflammation [34]. Both direct and developmental exposures to native steroids or EDC with steroid potential have been shown to influence adipocyte size [33, 35]. For instance, prenatal BPA exposed rats [36] and sheep [19] manifest large adipocytes. Furthermore, because adipose tissue is a site of steroid production and action [37], local changes in steroid biosynthetic machinery and steroid receptor expression can contribute to adipocyte differentiation and size.

As such insulin sensitivity of the metabolic tissues may be impacted by plethora of interactive regulators and adipocyte hypertrophy by the intracrine steroidal machinery. Utilizing female sheep exposed prenatally to BPA at levels spanning environmental to occupational exposure levels, the goal of this study was to understand the contributions of adiponectin, inflammatory / oxidative stress status, and lipotoxicity in metabolic tissues and steroidal machinery in the VAT in the development of IR and adipocyte hypertrophy.

METHODS

Animals

All animal procedures were approved by the Institutional Animal Care and Use Committee of the University of Michigan and are consistent with the National Institutes of Health's Guide for the Care and Use of Laboratory Animals. These studies were conducted at the University of Michigan Sheep Research Facility (Ann Arbor, MI) using Suffolk sheep breed. Breeder sheep (2–5-year-old) were acquired from local farmers, group housed and kept outdoors with access to shelter. Their maintenance, breeding, prenatal treatments and lambing were performed as described previously [38]. All animals including the control group were maintained together and any exposures to potential sources of phytoestrogens via diet were similar across treatment groups.

Prenatal BPA treatments

Pregnant sheep (n=8 to 13 per group) were randomly assigned to the different treatment groups (Figure 1). Treatment groups involved controls mothers that received only the vehicle (corn oil) and animals receiving 0.05, 0.5, or 5 mg/kg/·day (BPA_{low}, BPA_{med}, and BPA_{high}, respectively) of BPA (purity ≥99%, cat. no. 239658; Aldrich Chemical, Milwaukee, WI). BPA was dissolved in corn oil and administered daily through subcutaneous (sc) injections from days 30 through 90 of gestation (term: ~147 days). Daily sc administration of 0.5mg/kg/day dose of BPA from days 30–90 of gestation produced umbilical arterial levels of 2.62±0.52ng/ml of free BPA [39] on day 90 of fetal life, when measured in samples collected within an hour after the last injection of BPA. These levels are well within the range found in mid-gestation umbilical cord blood concentrations of free BPA (<LOD - 52.26 ng/mL) in a California based study [40] or of total BPA in term cord blood level (<LOD - 51.5 ng/mL) in Korean population [41]. The concentration of free BPA achieved in

cord blood in our study is consistent with levels achieved following maternal sc injection of 5mg/kg/day of BPA in sheep (10-fold higher dose than used in our study) from gestational days 28 to term in an earlier study (29.7±4.6 ng/ml) [42]. Although comparable levels of free BPA were detected, considering BPA was administered as single daily subcutaneous injections, it needs to be recognized that the levels in fetal circulation will likely fluctuate. Only female lambs were used for the study and these were weaned at ~8 week of age and provided with a maintenance diet consisting of 0.64 kg of corn, 0.64 kg hay·lamb−1·day−1, and 0.014 kg of supplement (36% crude protein) to prevent development of obesity. Wooden feeders with crossover metallic bars were used. Only one female offspring from each dam was utilized if twin pregnancies were involved. The number of female offspring available to study from each treatment group (one per mom) were control: 5; low BPA: 8; medium BPA: 10; high BPA: 5. The impact of prenatal BPA exposure on ovarian follicular dynamics, LH surge, insulin sensitivity, and adiposity utilizing the animals from this cohort have been previously reported [21, 43, 44].

Tissue Collection

Metabolic tissues were collected during the second breeding season at ~21 months of age following a 48h fast. Estrous was synchronized with two injections of prostaglandin F2α (PGF2α, 10 mg, i.m.; Lutalyse, Pfizer Animal Health, Florham Park, NJ) administered 11 days apart. Tissues were harvested 24 hours after the second PGF2α injection during the follicular phase much before the preovulatory increase in estradiol and the LH surge. The timing of tissue harvest was determined based on timing relationships from the preceding cycle where preovulatory increase in estradiol rise The timing of tissue harvest was determined based on and LH surge occurred namely ~40h and ~47h after second PGF2alpha injection in controls (prenatal BPA treatment had no effect on these timings) [43]. Blood samples from the jugular vein were collected in a heparinized tube and all animals euthanized by barbiturate overdose (Fatal Plus; Vortech Pharmaceuticals, Dearborn, MI). Circulating LH levels at the time of collection were well within the range of pulsatile LH secretion (data not shown). Liver was obtained from the tip of the left lobe, skeletal muscle from the vastus lateralis, and visceral adipose tissue (VAT) from the mesenteric fat surrounding the ventral sac of the rumen. Tissues were flash-frozen and stored at −80 °C until processed.

Inflammatory cytokines and antioxidant measures

Proinflammatory cytokines and antioxidant gene expression was assessed using SYBRgreen based real time RT-PCR on a BioRad myiQ iCycler instrument utilizing total RNA that was isolated, purified and reverse transcribed as described before [45]. Oligonucleotide primers for the genes under study were designed using Primer Express software (Life Technologies, Carlsbad, CA) and the sequences for the primers used are shown in Table 1. The relative amount of each transcript was calculated using the ΔΔCT method and normalized to the endogenous reference gene ribosomal protein L19 (RPL19).

The activity of antioxidant GSR was also determined in liver, muscle and VAT using a commercially available activity assay kit (Cayman chemicals, Ann Arbor, MI) as per manufacturer's recommendations.

Adiponectin

Plasma adiponectin was assessed using a previously published method [45, 46] using the bovine anti-adiponectin antibody kindly provided by Dr. Helga Sauerwein from University of Bonn, Germany. Plasma proteins were separated under reducing conditions to detect LMW forms and under non-reducing conditions to detect HMW forms and transferred to nitrocellulose membranes. Membranes were first stained with Ponceau to visualize the protein loading and incubated with primary antibody (Table 2) overnight at 4°C, washed and probed with HRP-tagged secondary antibody raised against the species in which primary antibody was generated. Adiponectin protein was detected by electrochemiluminescent (ECL) reaction using ProteinSimple FluorChem E system (San Jose, CA). Band densities for adiponectin from ECL reaction and albumin in the Ponceau stained membrane were determined using ImageJ software.

Steroidogenic enzymes and steroid receptors

Steroidogenic enzymes and steroid receptors were assessed in VAT by both real time RT-PCR and immunoblotting. Real time RT-PCR was performed as described above. For immunoblotting, whole-tissue protein extracts were prepared from tissue homogenates and equal amounts of protein (~40 μg) were resolved on SDS-PAGE, transferred onto a nitrocellulose membrane and protein content visualized and quantified as described for adiponectin with detection of GAPDH used as the loading control.

Oxidative stress measures

As markers of oxidative stress, levels of protein-bound oxidized tyrosine moieties, 3-nitrotyrosine (NY) and *o, o'*-dityrosine (DY) were quantified by isotope dilution liquid chromatography electrospray ionization tandem mass spectrometry (HPLC-ESI-MS/MS) as described previously [47] in tissue extracts from muscle and liver and lipidperoxidation marker Thiobarbituric Acid Reactive Substances (TBARS) utilizing commercially available colorimetric assay kit (Cayman Chemicals, Ann Arbor, MI) in tissue extracts from VAT, muscle and liver. For oxidized tyrosine quantification, tissue extracts were delipidated, precipitated and proteins hydrolyzed along with known amounts of isotopically labeled internal standards $^{13}C_6$-Y and $^{13}C_6$-NY, $^{13}C_6$-ClY, and $^{13}C_{12}$-*o, o'*-DY. The extracts were then subjected to solid-phase extraction and the amount of oxidized amino acids was quantified by HPLC-ESI-MS/MS. The levels of the oxidized amino acids in each sample were then normalized to the amino acid tyrosine content in the respective samples and expressed as the ratio of the oxidized product over the total tyrosine. Intraassay coefficients of variation for NY and DY were 8.47% and 8.16%, respectively. For TBARS assay, total protein of the tissue was isolated and assay carried out as per manufacturer's recommendation and Intra- and inter-assay coefficients were 5.6% and 10.2%, respectively.

Ectopic lipid accumulation

Lipid accumulation in liver were assessed by Oil Red O staining of cryosections and in both liver and muscle by measuring triglyceride content utilizing a commercially available assay kit as described before [45]. For oil red O staining, 3 slides containing sections chosen 50 microns apart were used and five regions corresponding to the 4 corners and center of each

section were imaged for determining the oil red O stain positive area. The area of oil red O positive stain to the total area of the imaged section was calculated using the Image Pro-Plus 3.0.1 system (Media Cybernetics, Rockville, MD) using a well-validated densitometrical methodology [48] as described previously [49]. Tissue triglyceride content was assessed in 100mg of tissue subject to lipid extraction as per the Bligh and Dyer method [50] with slight modifications as described before [45] using the Wako L-Type TG M kit (Wako Diagnostics, Mountain View, CA) as per manufacturer's recommendations. Each sample was assayed in duplicate and the intra- and inter-assay coefficients of variance were 3.32 ± 0.57 and 5.2 ± 0.73%, respectively. The measurable range for this assay is 1.1 to 2000 mg/dL. Plasma triglyceride content was assessed using a colorimetric assay kit (Cayman Chemicals, Ann Arbor, MI) as per manufacturer's recommendation. The intra- and inter-assay coefficients of variance were 2.14 and 6.71%, respectively with the detection limit of 0.5mg/dL

Statistical analysis

Heterogeneity of variance was tested with Bartlett's $\chi 2$ test and when required data were log-transformed. Changes in gene expression, markers of oxidative stress, and triglyceride content were analyzed using one-way ANOVA followed by Tukey's post hoc test using the Prism 6 software (GraphPad, La Jolla, CA) and the threshold for significance was set as $p \leq 0.05$. Data were also subjected to Cohen's effect size analysis [51–53]. ANOVA outcomes when significant (P<0.05) and Cohen's d value of >0.8 are presented in Figures with specifics in Figure legends.

RESULTS

Inflammatory markers

Prenatal BPA treatment did not lead to statistically significant changes in any of the inflammatory markers at all three sites studied. Results from Cohen's effect size analysis are presented below.

Liver: Effect size analysis lead to mixed outcomes depending on the inflammatory marker. This was reflected as 1) a reduction in the expression of several of the pro-inflammatory markers (interleukin 1 beta [*IL1B*; d = 0.90, 1.23 and 1.04], *IL6* [d = 1.22, 1.23 and 1.26] and chemokine (C-C) ligand 2 [*CCL2*; d = 0.97, 1.12 and 1.18]) for BPA_{low}, BPA_{med}, and BPA_{high} doses respectively, 2) an increase (d = 1.16) with tumor necrosis factor alpha (*TNF*) with only BPA_{low} dose, and 3) an increase in the macrophage marker *CD68* (d = 0.90, 1.23 and 1.04, respectively for BPA_{low}, BPA_{med}, and BPA_{high} doses) (Figure 2).

Skeletal muscle: Cohen's effect size analysis also pointed to an increase in 1) *IL6* (d = 0.97, 1.04 and 1.40 for the BPA_{low}, BPA_{med}, and BPA_{high} doses, respectively), 2) *IL1B* (d = 0.81, only with BPA_{med} dose), and 3) *CCL2* (d = 1.28 and 1.26) and the macrophage marker *CD68* (d = 1.36 and 1.00) expression with BPA_{low} and BPA_{med} doses (Figure 2).

VAT: Effect size analysis suggested increases in *IL1B* with all 3 doses (d = 0.90, 0.85 and 0.85 for the BPA_{low}, BPA_{med}, and BPA_{high} doses, respectively) and for *CCL2* with only the BPA_{high} dose (d = 2.02) (Figure 2).

Oxidative stress markers

Prenatal BPA treatment induced a significant increase in oxidized tyrosine moieties (NY but not DY content) at all doses in the liver and in BPA_{low} and BPA_{med} groups in the muscle (Figure 3). We have previously shown that both in circulation and VAT, prenatal exposure to BPA_{high} induces a significant increase in oxidative stress marker NY but not DY (Figure 3; mean levels from previous publication [21] shown in the grey boxed area for comparison). Effect size analysis pointed to an increase in lipid peroxidation marker, TBARS with the BPA_{high} dose in the liver (d = 2.05) and VAT (d = 1.83) and in the BPA_{low} and BPA_{med} doses in the muscle (d = 1.31 and 1.24, respectively) (Figure 3).

The expression of antioxidants in the liver and muscle was generally reduced with significant decrease in *GSR* mRNA expression with BPA_{med} and BPA_{high} doses in liver and both *GSR* and *SOD1* mRNA expression with BPA_{high} dose in the muscle (Figure 4). In contrast, in the VAT only antioxidant *GSR* showed a significant increase at BPA_{med} and BPA_{high} dose.

Effect size analysis pointed to decreases in expression of antioxidants 1) *GSR* mRNA with BPA_{med} (d = 1.38) and BPA_{high} (d = 1.39) doses in the liver, all doses (d = 1.53, 0.93 and 1.60 for the BPA_{low}, BPA_{med}, and BPA_{high} doses, respectively) in the muscle and BPA_{med} (d = 1.30) and BPA_{high} (d = 1.40) in the VAT, 2) *SOD1* mRNA with BPA_{low} (d = 1.02) and BPA_{high} (d = 0.85) in the liver, BPA_{high} (d = 5.77) in the muscle, and BPA_{med} (d = 1.10) and BPA_{high} (d = 0.91) in the VAT, and 3) *SOD2* mRNA with BPA_{low} (d = 1.13) in the liver and muscle (1.12). The GSR activity appeared as an increase (d = 1.24) with BPA_{high} dose in contrast to decreased mRNA expression in the liver. The GSR activity appeared as a decrease at BPA_{med} dose (d = 1.70) in the muscle consistent with its mRNA expression (Figure 4).

Lipotoxicity

Plasma triglyceride content did not change between the different BPA groups (Figure 5), however, a significant increase in both hepatic and muscular triglyceride content was evident at BPA_{med} and BPA_{high} dose groups (Figure 5) by ANOVA. Effect size analysis also showed an increase (d = 3.64) in hepatic triglyceride content in BPA_{high} dose group and BPA_{med} (d = 1.32) and BPA_{high} (d = 4.79) dose groups in the muscle. No significant changes in hepatic lipid content was evident when assessed by oil red O staining with any of the BPA treatment (Figure 5).

Adiponectin

The plasma LMW adiponectin levels were significantly reduced only in the BPA_{high} dose group (Figure 6). Effect size analysis pointed to a decrease (d = 0.87, 0.83 and 3.55 with BPA_{low}, BPA_{med}, and BPA_{high} doses, respectively) for LMW but not HMW adiponectin forms (Figure 6).

VAT steroidal milieu

Relative to androgens, prenatal BPA induced significant increase in *CYP17* mRNA with BPA_{high} dose. No change in CYP17 protein and *AR* mRNA expression was evident with any

of doses tested. Effect size analysis indicated a decrease (d = 1.18) in AR protein with the high BPA dose (Figure 7). Relative to estrogen, a significant increase in *CYP19* mRNA and a significant decrease in ESR2 protein at BPA_{med} and BPA_{high} doses were observed. Effect size analysis revealed an increase (d = 1.02) in CYP19 protein only with the BPA_{high} dose, *ESR1* mRNA at all doses (d = 0.76, 2.35, 2.91 for BPA_{low}, BPA_{med}, and BPA_{high} doses, respectively) and *ESR2* mRNA at BPA_{med} (d = 1.3) and BPA_{high} (d = 0.8) doses (Figure 7). No change in ESR1 protein was evident at any of the doses.

DISCUSSION

The findings from this study indicate that an increase in oxidative stress in the metabolic tissues, lipotoxicity in the liver and muscle, and systemic changes such as reduced adiponectin are all potential contributors of the IR status [19] of the prenatal BPA-exposed female sheep. Additionally, the increased estrogenic milieu with increased expression of estrogen biosynthetic enzymes and receptors in the VAT may have contributed to the adipocyte hypertrophy [19] observed in the prenatal BPA-exposed female sheep. Furthermore, many of the observed changes resulting from prenatal exposure to environmental to occupational levels of BPA-exposure followed a non-monotonic response curve, a finding consistent with other studies of BPA exposure [54–56]. The significance of these findings and their contribution to the IR status of these animals are discussed below.

Systemic Changes:

The observation of reduced LMW adiponectin in prenatal BPA-exposed female sheep is suggestive of hypoadiponectinemic state that is typical of metabolic conditions characterized with IR [23, 26]. Findings from this study and our previous findings of increased oxidative stress state [21] are therefore consistent with the IR state observed in prenatal BPA-exposed female sheep [19]. Low adiponectin levels such as that evidenced in this study are also a feature of perinatal BPA-exposed female mice [57] and male rats [58]. This contrasts with the lack of decrease in HMW adiponectin the form that promotes the insulin sensitizing action, low levels of which are reported in other conditions associated with IR [26]. The lack of change in plasma triglyceride content is surprising considered it is associated with IR status [23, 26]. In contrast, changes in TG were observed in perinatal and postnatal BPA exposed mice [59] and perinatal BPA exposed rats [60].

Liver:

Increase in oxidative state (characterized by increase in oxidative stress marker NY and downregulation of antioxidant gene expression) and triglyceride content in the liver from prenatal BPA-treated female sheep indicates that the negative mediators of insulin sensitivity are increased in the liver of prenatal BPA-treated sheep. This is consistent with the increases in hepatic lipid accumulation and oxidative stress observed in the male but not female Sprague-Dawley rats born to mothers exposed to BPA during gestation and lactation [61]. Hepatic lipid accumulation was manifested as increase in triglyceride content but not oil red O staining and this may be a function of oil red O stain only accumulating in neutral lipids [62]. A potential mechanism for hepatic lipid accumulation may involve altered expression of hepatic lipid metabolism related genes (adipose triglyceride, hormone sensitive and

lipoprotein lipases) as observed in perinatal BPA exposed mice [63]. The changes in inflammatory cytokines were evident as either increase in *TNF* (low dose only) and macrophage marker *CD68* or decrease for *IL1B*, *IL6* and *CCL2*, by effect size analysis only. Although these findings did not achieve statistical significance, the directionality of the changes by effect size analysis suggests that additional studies involving larger sample sizes are required to establish the potential involvement of liver inflammatory status to its IR status.

The hepatic oxidative stress and lipid accumulation in prenatal BPA-treated sheep parallel what is seen with the pathological condition, non-alcoholic fatty liver disease (NAFLD) [64, 65]. In this condition, a strong correlation has been reported between development of systemic and hepatic IR and accumulation of lipids in the liver [66]. Other studies pointing to amelioration of IR state with reversal of hepatic steatosis point to causal relationship between the two [67]. Additional support for the link between the two comes from studies in sheep treated during the same susceptibility window with native steroid, testosterone; these sheep not only manifested hepatic lipid accumulation, inflammation and oxidative stress that are characteristic of NAFLD [45] but also reduced phospho-AKT response to insulin stimulation [68] consistent with hepatic IR. Considering testosterone can be aromatized to estrogen and co-treatment with androgen antagonist partially reversed the hepatic lipid accumulation in this model [45], the programming of this defect is likely estrogen-mediated. These findings raise the possibility that changes in mediators of hepatic insulin sensitivity are likely accompanied by compromised hepatic insulin sensitivity also in the prenatal BPA (estrogen mimic)-treated sheep, a premise that remains to be tested.

Skeletal muscle:

The effects of prenatal BPA treatment on oxidative stress markers and lipid accumulation followed similar directionality as in the liver. This was reflected as increased muscle NY and a trend for TBARS content ($p = 0.08$ for low and medium dose, T test), reduced antioxidant gene expression and increased triglyceride accumulation. These findings indicate that negative mediators of insulin signaling are also increased in the skeletal muscle of prenatal BPA-treated female sheep. Although the muscle insulin sensitivity remains to be tested in this model, finding of reduced phospho-AKT response to insulin stimulation that accompanied increases in negative mediators of insulin sensitivity [68] in prenatal testosterone treated sheep raises the possibility that the increase in negative mediators of insulin sensitivity in the prenatal BPA-treated model may also be accompanied by compromised muscle insulin sensitivity. Such decreased AKT and glycogen synthase kinase 3 beta have been observed in the skeletal muscle from orally BPA administered mice [69]. Cohen's effect size analysis pointed to an increase in multiple inflammatory cytokines in the skeletal muscle. As these data did not reach statistical significance the relative contribution of inflammation in IR status of skeletal muscle needs to be examined with larger sample size.

VAT:

Adipocyte hypertrophy, inflammation and oxidative stress in the VAT are characteristic features of conditions associated with IR [70–72]. Findings from the present study of a trend

towards increased oxidative stress marker TBARS ($p = 0.06$, high dose only) and previously reported increased oxidative stress marker NY [21], negative mediators of insulin sensitivity, are consistent with the adipocyte hypertrophy [19] and IR [19], reported in this model. The increase in oxidative stress state manifested as lack of increase in GSR activity in BPA_{low} and BPA_{high} dose groups coupled with increases in oxidative stress markers (22) is also a feature consistent with conditions associated with IR [70]. Considering that the inflammatory status of the adipose tissue was evident as increase in expression of cytokines *IL1B* and *CCL2* (high dose) by effect size analysis only, confirmation of their proinflammatory status needs further investigation. The reduced circulating level of adiponectin, a major adipokine with anti-inflammatory role secreted by the white adipose tissue [26], is supportive of potential proinflammatory state.

Findings from this study of increase in the mRNA expression of *CYP19* in the VAT suggests that the steroidogenic biosynthetic machinery favors increase in estrogen biosynthesis. This coupled with increased ESR1 ($p < 0.05$ for medium and high dose, by t test) and the trend for decreased AR mRNA expression ($p = 0.09$ for high dose only by t test) indicates a predominant tendency for intracrine estrogenic milieu in the VAT of prenatal BPA-treated sheep. An estrogenic intracrine milieu is not consistent with this adipocyte hypertrophy phenotype [19] and contrasts with the observations in prenatal testosterone-treated female sheep where an increase in estrogenic milieu in the VAT accompanies smaller adipocytes [73, 74]. Estrogens have been shown to reduce adipocyte cell size by decreasing adipocyte lipid storage and increasing lipolysis [33, 75]. The increase in liver and muscle lipid accumulation in prenatal BPA-treated sheep coupled with the intracrine estrogenic milieu is suggestive of increased lipolysis. Therefore, the increase in adipocyte size in the prenatal BPA-treated sheep may be independent of estrogen's effect at the level of lipid storage and may occur at the level of adipocyte differentiation; enhanced differentiation of preadipocytes have been observed in sheep exposed to BPA during gestation [76].

Conclusions

In summary, prenatal BPA treatment increases the negative mediators of insulin sensitivity such as oxidative stress and lipotoxicity and reduces positive mediators of insulin sensitivity such as adiponectin and antioxidants in the circulation and the metabolic tissues of the prenatal BPA-treated female sheep (Table 3). While there is the possibility of involvement of inflammatory pathways in the muscle and VAT, these findings need to interpreted with caution considering that such changes were only based on effect size analysis. Although the findings relative to the inflammatory status of tissues studied are inconclusive and a limitation of this study, the directionality of changes in inflammatory cytokines suggested by effect size analysis is in line with other significant changes in negative mediators of insulin sensitivity (oxidative stress and lipotoxicity) that are generally associated with development of IR at the peripheral as well as tissue level [77]. Additional studies are warranted to conclusively prove the involvement of inflammatory markers in prenatal BPA induction of tissue-specific IR. Changes in negative mediators of insulin sensitivity in muscle, liver and adipose tissues is postulated to be the common cause of IR associated with metabolic conditions such as type 2 diabetes, obesity, cardiovascular diseases, NAFLD, etc. [78, 79], a feature also seen in tissue-specific conditional knockouts of insulin receptor (an animal

model with loss of insulin sensitivity) [80]. Whether these changes in negative mediators of insulin sensitivity accompanies reduced insulin signaling at the tissue level, such as that observed in prenatal testosterone-treated sheep [77] needs to be determined. Additional studies are also warranted to understand the mechanisms via which gestational exposure to BPA program these tissue-specific changes. The finding of increased hepatic DNA methylation and development of IR during adulthood [81] in perinatal BPA treated rats suggests that epigenetic modifications could be the mediaries. While this study focused on the changes in mediators of insulin sensitivity, recent finding that gestational BPA alters fetal thyroid function [82], important regulators of metabolic functions including hepatic steatosis [83], suggests disruptions in thyroid function may also be a contributor to the prenatal BPA-induced metabolic disruptions.

Although these studies were carried out in a polygastric species, findings have the potential to be of translational relevance to human, a monogastric species. There are several similarities between the two species relative to the metabolic focus of the study namely low blood glucose levels due to fasting, postprandial changes in blood glucose / insulin, tissue-level insulin action, and development of insulin resistance with increased adiposity, [84–87]. Additionally, fetal developmental timeline, precociality at birth, diurnal behavioral pattern, and pathways that regulate appetite, energy balance, and adipogenesis, and distribution of brown/beige fat in white adipose tissues are also similar between sheep and humans [20, 88–91] thus making them valuable translational models. However, as these polygastric animals predominantly use non-esterified free fatty acids (NEFFA) as a source of energy, further studies are required to evaluate if disruptions in homeostasis of NEFFA and their associated regulatory pathways have any role in the manifestation of metabolic disruptions.

In summary, findings from this study provide evidence in support of tissue-specific developmental impact of prenatal BPA treatment, at environmentally relevant levels, on metabolic mediators of insulin sensitivity.

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Highlights

- Prenatal BPA disrupts mediators of insulin sensitivity in female sheep
- Prenatal BPA induces oxidative stress status in metabolic tissues
- Prenatal BPA induces dyslipidemia and lipotoxicity in the liver and muscle
- Elevated negative mediators of insulin sensitivity underlie prenatal BPA effects

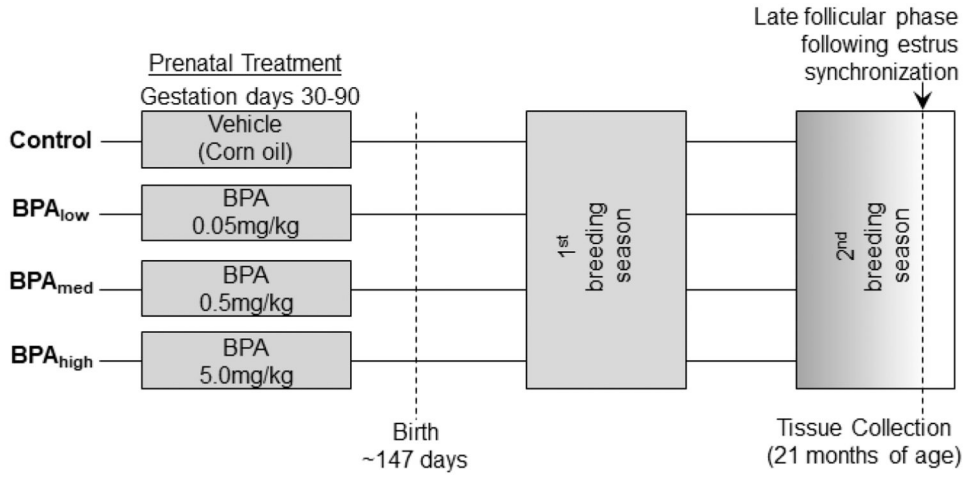

Figure 1:

Schematic showing the study design with the time and duration of treatment with different doses of BPA and time of tissue collection.

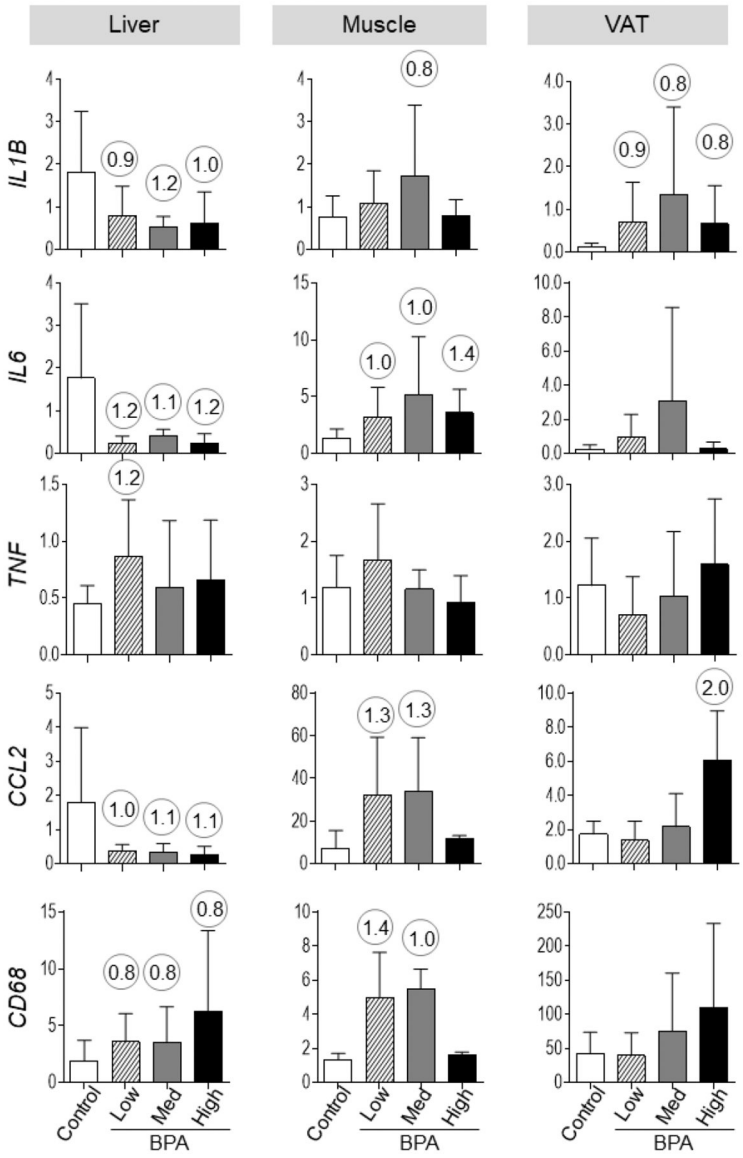

Figure 2:

Mean ±SEM of proinflammatory cytokines (mRNA expression of *IL1B*, *IL6*, *TNF*, *CCL2*) and macrophage marker (*CD68)* expression in control, BPA_{low}, BPA_{med} and BPA_{high} dose prenatal BPA-treated groups in the liver (left), muscle (middle) and VAT (right panel). Numerical superscripts are Cohen's d values ≥ 0.8 comparing control and different dose groups of BPA. None of these changes achieved statistical significance.

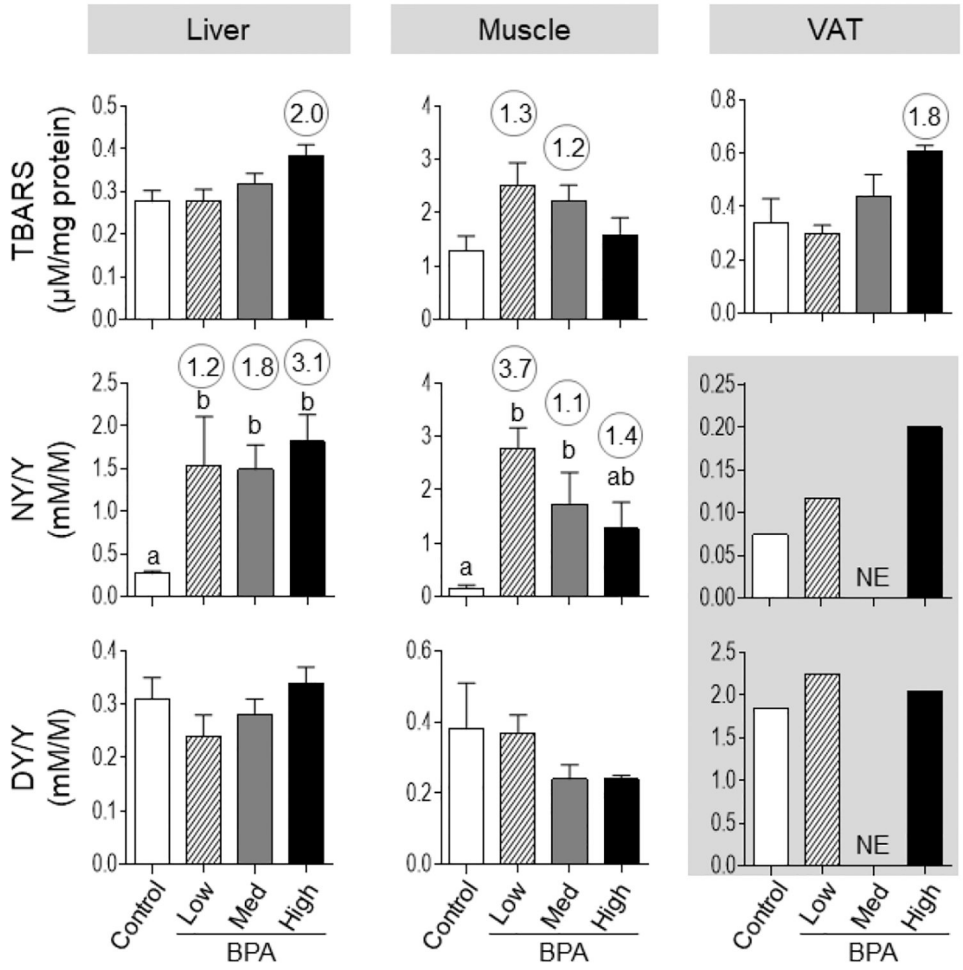

Figure 3:

Mean ± SEM tissue TBARS and oxidized tyrosine concentrations in control, BPA_{low}, BPA_{med} and BPA_{high} dose prenatal BPA-treated groups in the liver (left), muscle (middle) and VAT (right panel). Numerical superscripts are Cohen's d values ≥ 0.8 comparing control and different dose groups of BPA. Mean values of oxidized tyrosine concentrations in VAT that is previously published [21] are shown in the grey area (NE = Not Examined). Differing letters in histograms (NY/Y in liver and muscle) indicate statistically significant differences; a vs. b: $p<0.05$; ab not different from a or b.

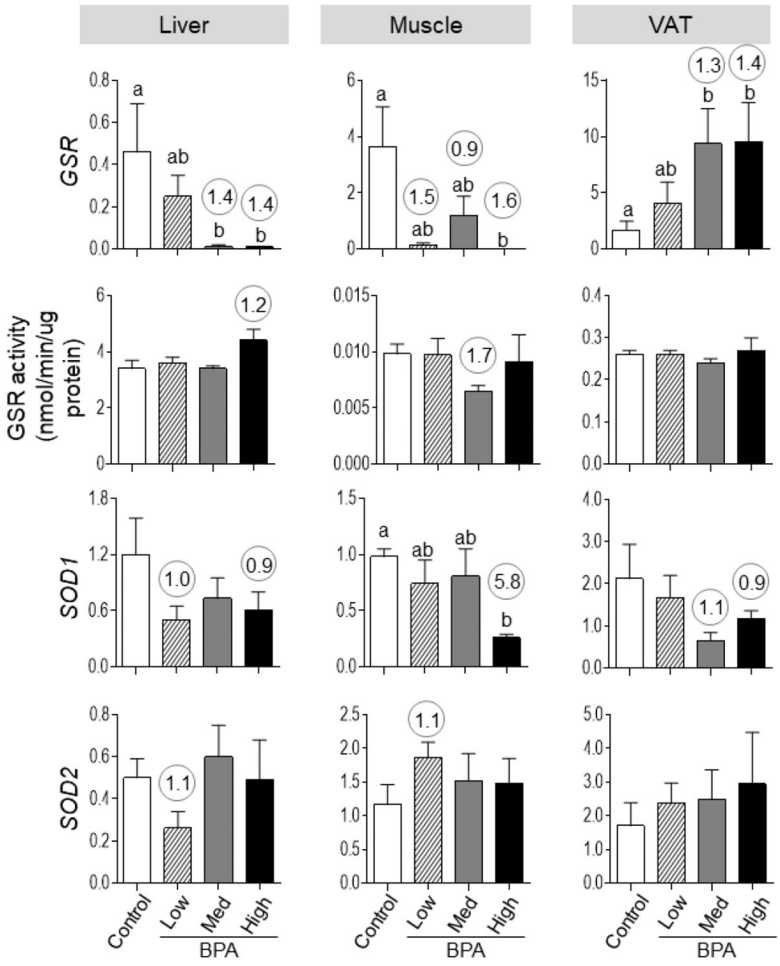

Figure 4:

Mean ± SEM antioxidant [*GSR* (top row), *SOD1* (middle-lower row) and *SOD2* (bottom row)] expression in the liver (left), muscle (middle) and VAT (right panel) tissues of control, BPA_{low}, BPA_{med} and BPA_{high} dose prenatal BPA-treated groups. Mean ± SEM of enzymatic activity of GSR are also shown for these different tissues and treatment groups in middle-top panel. Differing alphabets above the histogram (GSR in liver, muscle and VAT, SOD1 in muscle) indicate statistically significant differences by ANOVA (a vs. b: $p<0.05$; ab not different from a or b) and numerical superscripts are Cohen's d values ≥ 0.8 comparing control and different dose groups of BPA.

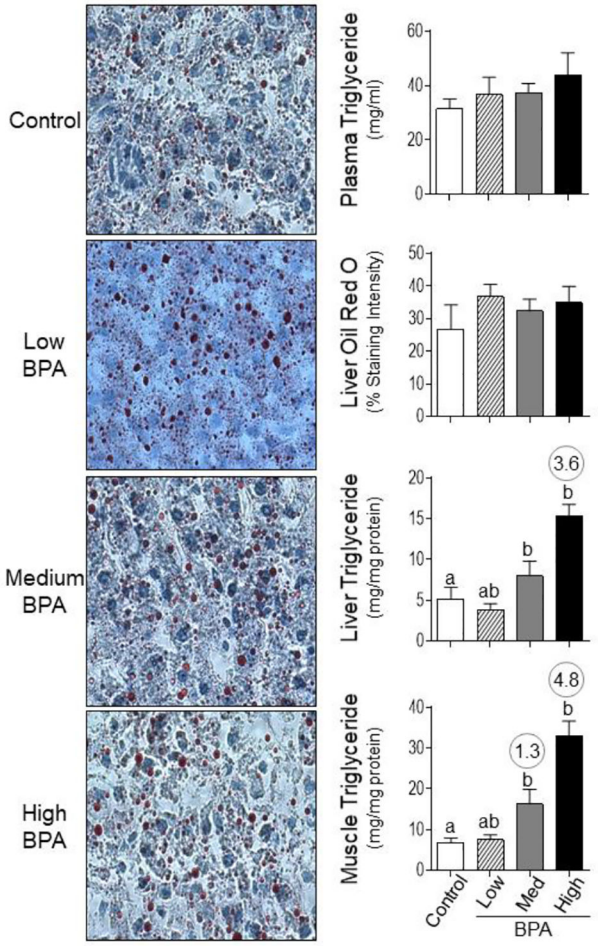

Figure 5:

Examination of lipid accumulation in plasma, liver and muscle from control, BPA_{low}, BPA_{med} and BPA_{high} dose prenatal BPA-treated groups. The cryosections of liver stained with oil red O and imaged for assessment of staining intensity as described in the methods are shown in the left panels (Bar = 25μm). On the right are shown mean ± SEM of the percent staining of oil red O in liver (right middle-top) and triglyceride content in plasma (right top), liver (right middle-bottom) and muscle (right bottom). Differing letters in histograms (liver and muscle triglyceride) indicate statistically significant differences by ANOVA (a vs. b: $p<0.05$; ab not different from a or b) and numerical superscripts are Cohen's d values ≥ 0.8 comparing control and different dose groups of BPA.

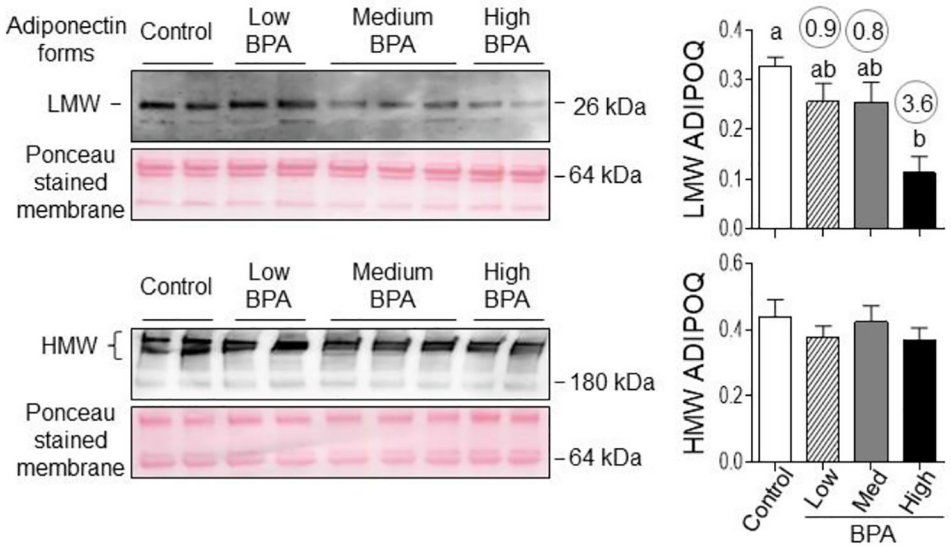

Figure 6:

Plasma levels of low (LMW) and high (HMW) molecular weight forms of adiponectin in control, BPA_{low}, BPA_{med} and BPA_{high} dose prenatal BPA-treated groups. Top panels show representative blots under reducing conditions (left, LMW) and non-reducing conditions (right, HMW forms) along with Ponceau staining for protein loading. Right panels show mean ± SEM of density ratios between LMW (top) and HMW (bottom) forms of adiponectin with the 64 kDa protein from the Ponceau stained membrane. Numerical superscripts are Cohen's d values ≥ 0.8 comparing control and different dose groups of BPA. Differing letters in histograms (LMW ADIPOQ) indicate statistically significant differences by ANOVA (a vs. b: $p<0.05$; ab not different from a or b).

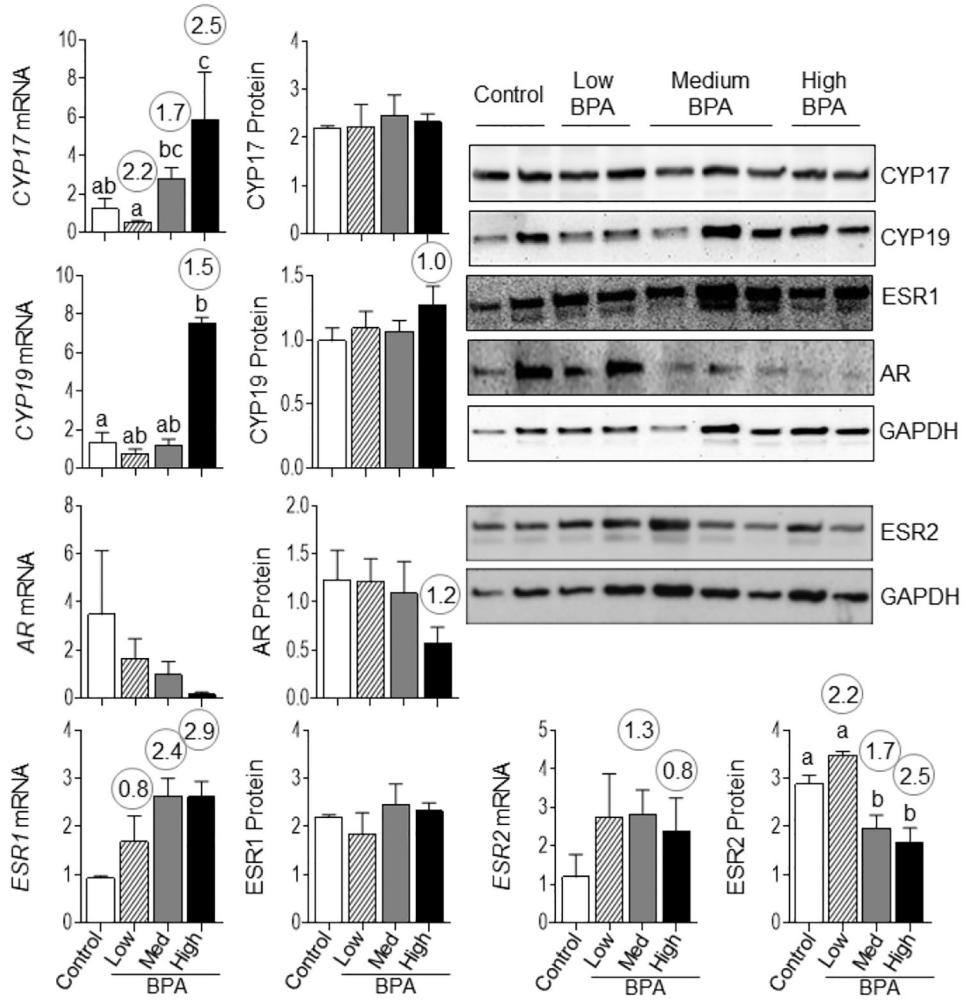

Figure 7:

Changes in steroidogenic enzymes CYP19 and CYP17, and steroid receptors ESR1, ESR2 and AR mRNA and protein expression in VAT from control, BPA_{low}, BPA_{med} and BPA_{high} dose prenatal BPA-treated groups are shown. Gene expression was quantified by RT-PCR and presented as mean ± SEM of fold changes of treated vs. control samples. Protein content was determined by immunoblotting and mean ± SEM of ratio of densitometric values for the respective protein to GAPDH are plotted. Representative immunoblots are shown in the right side of the panel. Differing alphabets above the histogram indicate significant changes by ANOVA and numerical superscript are Cohen's d values ≥ 0.8 comparing control and different dose groups of BPA. Differing letters in histograms (*CYP17* and *CYP19* mRNA) indicate statistically significant differences by ANOVA (a vs. b: $p<0.05$; ab not different from a or b).

Table 1:

Sequence of the primers used for real time RT-PCR

Gene ID	Forward Primer	Reverse Primer	Accession Number
IL1B	CGAACATGTCTTCCGTGATG	GAAGCTCATGCAGAACACCA	NM_001009465.2
IL6	ACATCGTCGACAAAATCTCTGCAA	GCCAGTGTCTCCTTGCTGTTT	NM_001009392.1
TNF	ACACCATGAGCACCAAAAGC	AGGCACAAGCAACTTCTGGA	XM_005223596.3
CCL2	CCAGCAGCAAGTGTCCTAAAG	GGCTTTGGAGTTTGGTTTTTC	XM_004012471.2
CD68	CAGGGGACAGGGAATGACT	CCAAGTGGTGGTTCTGTGG	NM_001045902.1
GSR	CTGGAAGAGTTGCCTCGCC	TCATTATTGATGTCTTAGAACCCAGG	XM_015104590.1
SOD1	ATCATGGGTTCCACGTCCA	CATGCCTCTCTTCATCCTTTGG	NM_001145185.1
SOD2	CGCTGGAGAAGGGTGATGTC	CAGATTTGTCCAGAAGATGCTGTG	NM_001280703.1
CYP17	AGACATATTCCCTGCGCTGA	GCAGCTTTGAATCCTGCTCT	XM_012102863.2
CYP19	TGGCCTGGTGCGCATGGT	TGCGCCGCATGAGGGTCAAC	NM_001123000
ESR1	TCGTCTCGGTTCCGTATGATG	GACAGAAATGTGTACACCCCAGAAT	AY033393.1
ESR2	GCCGACAAGGAACTGGTACAC	CCACGAAGCCCGGAATCT	NM_001009737.1
AR	GCCCATCTTTCTGAATGTCC	CAAACACCATAAGCCCCATC	XM_001253942
RPL19	CCTTGGCTCGCCGGA	CATGTGGCGGTCAATCTTCTTA	XM_012186026.1

Table 2:

Antibodies used for immunoblot analysis

Peptide/Protein Target	Antigen Sequence (if Known)	Name of Antibody	Manufacturer, Catalog Number, and/or Name of Individual Providing the Antibody	Species Raised in; Monoclona l or Polyclonal	Dilution Used	RRID
Bovine Adiponectin		Bovine anti-adiponectin	Dr. Helga Sauerwein (University of Bonn, Germany)	Rabbit; polyclonal	1:1000	AB_2650604
CYP17		Rabbit anti-17α-hydroxylase	Dr. Walter L Miller (University of California, San Francisco)	Rabbit; polyclonal	1:1000	AB_2732802
CYP19		Aromatase Polyclonal Antibody	Thermo Fisher Scientific, PA1–21398	Rabbit; polyclonal	1:1000	AB_2088676
AR		AR (N-20)	Santa Cruz Biotechnology, SC-816	Rabbit; polyclonal	1:1000	AB 1563391
ESR1		ERα (clone 1D5)	Thermo Scientific, MS-354	Mouse; monoclonal	1:500	AB 61341
ESR2		Estrogen receptor beta (ER 2) antibody	Dr. Benita S Katzenellenbog en (University of Illinois)	Mouse; monoclonal	1:1000	AB_2722105
GAPDH		GAPDH (14C10)	Cell Signaling, 3683	Rabbit; monoclonal	1:1000	AB 1642205

Table 3:

Summary of tissue-specific changes in the mediators of insulin sensitivity in prenatal BPA-treated female sheep

Tissue	Adiponectin	Inflammatory State	Oxidative Stress	Lipotoxicity	Steroidal Milieu	Predicted Effect on Insulin Sensitivity
Systemic	↓	NE	↑[#]	-	NE	**Resistant**
Liver	-	?	↑	↑	NE	**Resistant**
Skeletal Muscle	-	Inflammatory[*]	↑	↑	NE	**Resistant**
VAT	NE	Inflammatory[*]	↑[#]	NE	Estrogenic[*]	**Resistant**

NE= not examined; Arrows indicate significant outcomes.

[*] Effect size analysis point to this outcome- requires validation with larger sample size.

[#] Previously reported in Veiga-Lopez et al 2015 (21)