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Recovery from Developmental Nonylphenol Exposure is Possible I. Male

Ling-Ling Chang[1*], Wan-Song A. Wun[2], and Paulus S. Wang[3, 4, 5,6*]

1. Department of Chemical and Materials Engineering, Chinese Culture University, Taipei 11114, Taiwan, Republic of China
2. Obstetrical and Gynecological Associates, P. A., Houston, TX 77054, U.S.A.
3. Department of Physiology, School of Medicine, National Yang-Ming University, Taipei 11221, Taiwan, Republic of China
4. Department of Medical Research and Education, Taipei Veterans General Hospital, Taipei 11217, Taiwan, Republic of China
5. Graduate Institute of Basic Medical Science, Ph.D. Program for Aging, China Medical University, Taichung 40402, Taiwan, Republic of China
6. Department of Biotechnology, Asia University, Taichung 41354, Taiwan, Republic of China

* E-mail of the corresponding author: llchiang@faculty.pccu.edu.tw

Abstract
Nonylphenol (NP) is an environmental endocrine-disrupting chemical (EDC) that has been detected in human cord blood and milk. It is unavoidable that human fetus and infant exposure to this environmental contaminant. According to "fetal origins adult disease" hypothesis, the biological impact and healthcare will encounter unavoidable impact. We previously observed that developmental NP exposure led to increased body weight, elevated plasma ACTH, higher production and concentrations of corticosterone and aldosterone, and more 11β-hydroxysteroid dehydrogenase I (11 -HSD1) expression/activity during the first generation at the adult stage. With these phenomena, is human going to evolution to a heavier with metabolic syndrome state or back to "default state" after generation(s) of hygienic up. This study addressed the possibility of recovering from NP exposure.
Female rats were timed-mated in this experiment. Throughout gestation and lactation, one group of pregnant females was given a 2 μg/ml NP drinking solution and another group was given water. The litters were marked as first-generation F_1 NP or F_1 Veh offspring. At approximately 13 weeks of age, the F_1 females were timed-mated with non-sibling F_1 males from identical prenatal and neonatal treatment groups. The females were not manipulated in any way. The resulting litters were designated as the second-generation F_2 NP or F_2 Veh offspring. At 13 weeks of age, the male offspring from each F_1 and F_2 group were decapitated. The experimental results showed that NP exposure resulted in F_1 offspring hyperadrenalism and weight increases. These effects were not observed in the F_2 offspring. The F_2 generation status was set back to the 'default' stage, which shows the elevated body weight and hyperadrenalism returned to normal. This study indicates developmental exposure to NP results in life long impact. The recovery to "default state" is possible only after generation(s) suffer with expensive healthcare burden.
Keywords: NP, developmental exposure, 11 -HSD1, body weight, hyperadrenalism

1. Introduction

The global trend in human body weight continues to increase. Because of its relatively quick onset, the obesity epidemic may be caused by environmental rather than genetic factors (Skinner et al., 2011). The exponential production of synthetic chemicals by developing and developed countries has been associated with overweight and obese adults (Baillie-Hamilton, 2002). The United Nations Environmental Programme and the World Health Organization (WHO) recently described the obesity epidemic as a 'global threat' and called for more research on environmental endocrine-disrupting chemicals (EDCs) (Bergman et al., 2013). The National Institute of Environmental Health Sciences (NIEHS) and the Environmental Protection Agency also organized scientific meetings to discuss the link between EDCs, type 2 diabetes, and obesity (Thayer et al., 2012). Some EDCs can disrupt adipogenesis, weight and energy balance, and they are classified as obesogens (Grün and Blumberg, 2006; Janesick and Blumbert, 2012; Latini et al., 2010). In the United States, the prevalence of obesity in males and females is approximately 35% (Flegal et al., 2012). If overweight (BMI 25−29.9) and obesity (BMI $\geq$ 30) are both taken into account, approximately two-thirds of adults in the United States have an unhealthy BMI (Flegal et al., 2010, 2012). The consequence of being overweight or obese is an increase in the incidence of metabolic syndrome (i.e., insulin resistant diabetes, hypertension, and cardiovascular disease), reduced life span and poorer quality of life (Latini et al., 2010). Obese parents usually have overweight/obese children (Khashan and Kenny, 2009; McLoone and Morrison, 2012; Steffen et al., 2009). Overweight or obese children are more likely to become obese adults (Charney et al., 1976; Mijailović et al., 2001). Human health appears to be headed

in a one-way downward spiral and the demand for health care continues to grow. According to a 2008 WHO report, approximately 60% of deaths worldwide are due to metabolic syndrome. West-Eberhard (2003) described the concept of genetic assimilation, which states that an adaptive trait may be inherited if the environment remains stable for many generations. This could be a nightmare if the predisposition to obesity and metabolic syndrome becomes the 'norm'. Is civilized society destined to have large amounts of obesity and metabolic syndrome? Is there a way to recover from the EDC effect?

Nonylphenol (NP) is a nonylphenol ethoxylate degradation product as well as a widely used non-ionic surfactant in agriculture and.industry. It is also used in household products such as detergents, cleaners, indoor pesticides, cosmetics, and food packaging (US EPA RIN 2070-ZA09). Chronic human adrenal corticoid hormone elevation can lead to Cushing's syndrome, which is associated with abdominal obesity, glucose intolerance, and hypertension. These symptoms are similar to those of metabolic syndrome. In our previous study, NP stimulated glucocorticoid and mineralocorticoid release from cell cultures (Chang et al., 2010, 2012a). In an in vivo model, developmental (in utero and neonatal animals, F_1 generation) exposure to an environmentally relevant concentration of NP resulted in adrenal gland activity augmentation and increased body weight in adult male rats (Chang et al., 2012b). This model was intended to explore the possibility of reversing the decline in health conditions. EDC is believed to impact mediates through epigenetic modification (Skinner et al., 2010a). During periods of embryogenesis and gametogenesis, epigenetic modifications occur on a genome-wide level to demolish and re-build (Li, 2002; Morgan et al., 2005; Reik, et al., 2001; Seisenberger et al., 2013). The central hypothesis is that EDC epigenetic modifications are also erased/demolished and genetic activity is reset to a 'default' stage for the next generation, i.e., the F_2 generation. The F_2 generation may then be free from EDC impact and predisposition to obesity and metabolic syndrome.

2. Materials and Methods

2.1. Chemicals

NP was purchased from Fluka (Buchs, Switzerland) and a 0.425 M stock solution was prepared in methanol. Diethyl ether was purchased from Merck (Whitehouse Station, NJ, USA). All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA). [^{3}H]-Aldosterone and [^{3}H]-Corticosterone were sourced from Amersham Life Science Limited (Buckinghamshire, UK). Dr. Douglas M. Stocco (Department of Cell Biology and Biochemistry, Texas Tech University Health Sciences Center, Lubbock, TX, USA) generously provided the anti-StAR antibody. Anti-PPARγ anti-11β-HSD1 and anti-GR antibodies were purchased from Cayman Chemical (Ann Arbor, MI, USA), Abcam (Cambridge, UK) and Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA), respectively. Anti-mouse and anti-rabbit IgG peroxidase-conjugated secondary antibodies were purchased from ICN Pharmaceuticals, Inc. (Aurora, OH, USA).

2.2. Animals

Female 2-month-old Sprague-Dawley rats (n = 6) weighing 250–300 g were provided by the National Yang-Ming University and housed in a temperature-controlled room (22 ± 1°C) with a 14 h (light):10 h (dark) photoperiod. Food and water were provided ad libitum. The rats were time-mated following acclimatization. A single virgin female rat was housed with a male rat in a breeding cage until a vaginal plug was noted (designated F_0, day 0 of pregnancy). The females were then caged separately from pregnancy until delivery, which occurred between days 20 and 22. One group of pregnant females (n = 3, NP mothers) was given 2 μg/ml NP in water, and a second group of pregnant females (n = 3, Veh mothers) was given water throughout gestation and lactation. The litters were sexed and determined to be first generation F_1 NP or F_1 Veh offspring at birth. At approximately 13 weeks of age, F_1 females weighing approximately 230−280 g were time-mated with nonsibling F_1 males from identical prenatal and neonatal treatment groups. Females were caged separately during pregnancy and lactation and were not manipulated in any way. These litters were designated the second generation F_2 NP or F_2 Veh offspring. There were 10−12 pups per litter, and these offspring were housed with their mothers during lactation. At approximately 3 weeks of age, the offspring were weaned and caged in single-sex groups. Food and water were provided ad libitum. The number of male offspring in the same group (NP or Veh group) totaled 2−3 per cage, and the number of female offspring from the same group included 5−6 per cage. All the offspring were housed with their mothers in the same temperature-controlled room (22 ± 1°C) with photoperiods of 14 h (light):10 h (dark) with the light turned on at 6:30 a.m. Male rats were sacrificed at 13−14 weeks of age. To avoid disturbing their adrenal rhythms, rats were sacrificed promptly at 7:00 a.m. The pattern of sacrifice was completed with sequential alternations in the examination groups, i.e., the Veh group followed by the NP group followed by the Veh group, etc., or vice versa. Processing one rat usually required approximately 10−15 minutes, i.e., to collect their blood and tissues, and approximately 3−3.5 hours to complete the sample collection (usually before 10:30 a.m.). Trunk blood was collected and plasma was stored at -20°C until analysis. Liver, adipose tissue, and adrenal glands were immediately dissected and frozen in liquid nitrogen.

All animal protocols used in this study were approved by the Institutional Animal Care and Use Committee of

the National Yang-Ming University. Rats were humanely sacrificed by decapitation. All animals were treated in compliance with the Principles of Laboratory Animal Care and the Guide for the Care and Use of Laboratory Animals as published by the National Science Council, Taiwan, R.O.C.

2.3. Tissue protein extraction

Liver and adipose tissues were homogenized on ice in homogenization buffer (65 mM Tris pH 7.4 containing 154 mM NaCl, 1% NP-40 (vol/vol), 6 mM sodium deoxycholate, 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM phenylmethylsulfonyl fluoride (PMSF), 5 mg/l aprotinin, 5 mg/l leupeptin, 5 mg/l pepstatin, 1 mM sodium orthovanadate, 1 mM sodium fluoride, and 1% proteinase inhibitor cocktail (vol/vol)). Adrenal gland tissues, including the adrenal capsule and cortex, were homogenized in 2.5 mM Tris pH 7.8 containing 1.5% Na-lauroylsarcosine (wt/vol), 1 mM EDTA, 0.68% PMSF (wt/vol), and 2% proteinase inhibitor cocktail (vol/vol). Homogenates were centrifuged at 13,000 g for 30 min at 4°C, and their supernatants were then collected. Protein concentrations were measured by Bradford assay (Bradford, 1976) with bovine serum albumin (BSA) as a standard.

2.4. Enzyme activity assays

The 11β-HSD1 activity assay was performed as previously described (Eijken et al., 2005; McCormick et al., 2006), and it was used to quantify the production of corticosterone from 11-dehydrocorticosterone by radioimmunoassay (RIA). In brief, 20 µl of 50 mM sodium phosphate buffer pH 7.4 containing 1 mM EDTA, 1 M NaCl, 40% glycerol (vol/vol), and 0.4% Triton X-100 (vol/vol) was pre-incubated with 10 µl each of 2.4 mM nicotinamide adenine dinucleotide phosphate (NADPH) and 1 mM 11-dehydrocorticosterone for 3 min at 37°C. The reaction was started by adding 60 µl of tissue extract (0.5−1.0 mg protein), and the tubes were incubated for 1 h at 37°C. The reaction was terminated by submerging the tubes in ice. Controls consisted of blank tubes in which neither NADPH nor 11-dehydrocorticosterone was added.

A P450scc activity assay was used to quantify pregnenolone production from 25-OH-cholesterol by using an ELISA. In brief, one-half of the adrenal cortex in 1 ml of KRBGA (Krebs-Ringer bicarbonate buffer containing 3.6 mmol K^+/l, 11.1 mmol glucose/l, and 0.2% BSA (wt/vol)) was pre-incubated for 1 h at 37°C. Following pre-incubation, half the adrenal cortex was transferred to another tube containing 1 ml of KRBGA, 10 µM 25-OH-cholesterol and trilostane (an inhibitor of 3β-HSD activity), and incubated for 1 h at 37°C. The reaction was terminated by submerging the tubes in ice. An 11β-hydroxylase activity assay was used to quantify corticosterone production from deoxycorticosterone by using an RIA. In brief, one-half of the adrenal cortex in 1 ml of KRBGA was pre-incubated for 1 h at 37°C. After pre-incubation, half the adrenal cortex was transferred to another tube containing 1 ml KRBGA and 1 µM deoxycorticosterone, and then incubated for 1 h at 37°C. The reaction was terminated by submerging the tubes in ice. An aldosterone synthase activity assay was used to quantify the production of aldosterone produced from corticosterone by using an RIA. In brief, a capsule with half the adrenal gland in 1 ml of KRBGA was pre-incubated for 1 h at 37°C. After pre-incubation, the adrenal gland capsule was transferred to another tube containing 1 ml of KRBGA and 0.2 µM corticosterone, and then incubated for 1 h at 37°C. The reaction was terminated by submerging the tubes in ice. The specific activity was expressed as nanograms of steroid hormone formed per hour per microgram of protein.

2.5. Corticosterone in tissue homogenates

Adipose tissue was excised and washed in ice-cold isolation media (10 mM Tris-HCl pH 7.4 containing 0.25 M sucrose, 1 mM EDTA, and 0.25 mg BSA/ml) to remove the blood cells (Shukla et al., 2000; Swegert et al., 1999). After mincing the adipose tissue with scissors, the 10% (wt/vol) homogenates were prepared with a homogenizer. Homogenates were centrifuged at 13,000 *g* for 20 min at 4°C, and the supernatants were collected and stored at -20°C for diethyl ether extraction and corticosterone measurement by RIA.

2.6. Western blot analysis

Liver (100 µg), adipose (200 µg), and adrenal gland (100 µg) supernatants were subjected to western blot analysis (Lo et al., 2000) in order to detect 11β-HSD1 in the liver, PPARγ in the adipose tissue, GR in the liver and adrenal gland, or steroidogenic acute regulatory (StAR) protein in the adrenal gland. Either β-actin or GAPDH (β-actin was the internal control for the liver and adrenal gland, GAPDH was the adipose control) were used as internal controls. Samples containing equal amounts of protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with 10% *T* polyacrylamide gels for western blotting. Proteins were detected with specific antibodies and visualized by enhanced chemiluminescence using ECL western blotting detection reagents (Amersham Pharmacia Biotech, Buckinghamshire, UK). The resulting images were captured with an LAS-4000 luminescent image analyzer (Fuji-Film, Stamford, CT, USA), and the protein bands were quantified with a MultiGauge program (Fuji-Film). The 11β-HSD1, GR, and StAR protein levels were corrected against β-actin protein levels, and the PPARγ protein level was corrected against the GAPDH protein level.

2.7. Corticosterone, aldosterone, ACTH, and prolactin RIAs

Corticosterone and aldosterone in plasma or adipose supernatants were first extracted with diethyl ether. In brief,

1 ml of plasma or adipose supernatant was mixed with 10 ml of diethyl ether for 30 min at room temperature to extract steroid hormones from the inorganic phase. The sample was then left undisturbed for 15 min to separate the diethyl ether phase from the inorganic phase. The inorganic phase was snap-frozen in a dry ice-acetone bath. The diethyl ether phase was transferred into a new glass tube and evaporated, allowing hormones to be deposited on the tube wall. Hormones were resuspended in 1 ml of assay buffer and the tube was vortexed for 30 min.

An RIA was used to quantify the corticosterone level in the plasma or media as previously described for PSW#4−9 antiserum (Lo et al., 1998). The sensitivity of corticosterone RIA was 5 pg/tube, and the intra- and inter-assay coefficients of variation were 3.3% (n = 5) and 9.2% (n = 4), respectively. An RIA was used to quantify the aldosterone concentration in plasma or media as previously described for JJC-088 antiserum (Kau et al., 1999). The aldosterone RIA sensitivity was 4 pg/tube. The intra- and inter-assay coefficients of variation were 3.9% (n = 5) and 8.2% (n = 4), respectively.

Plasma ACTH levels were assayed by using a gonadotropin-releasing hormone RIA as previously described (Wang et al., 1983) with minor modifications from reagents supplied by NIDDK (Bethesda, MD, USA). Carrier-free ^{125}I was obtained for hormone iodination from PerkinElmer (Waltham, MA, USA). Human ACTH (h-ACTH) was iodinated with chloramine-T (Sigma Chemical Co.) as the oxidizing agent (Greenwood et al., 1963). The plasma ACTH level was measured by combining 200 µl of plasma with 100 µl of assay buffer (PBS pH 7.4 containing 0.05 M EDTA). This mixture was then incubated with 200 µl of anti-h-ACTH (diluted 1:1,000) at 4°C, 100 µl [^{125}I] ACTH and 50 µl of a secondary goat anti-rabbit antibody were added on the second and third days, respectively, after which the mixtures were incubated for an additional 48 h at 4°C. At the end of the incubation, 2 ml of PBS was added, and the assay tubes were centrifuged at 1000 *g* for 30 min at 4°C. The supernatants were discarded, and radioactivity was measured with a gamma counter (1470 Wallac Wizard Gamma Counter, PerkinElmer). The ACTH RIA sensitivity was 20 pg/tube.

Plasma prolactin (PRL) was measured with an RIA by using a protocol provided by the NIDDK Hormone Distribution Program. NIDDK-rPRL-RP-3, NIDDK-rPRL-I-6, and NIDDK-anti-rPRL-IC-5 were used in the PRL RIA. The assay sensitivity was 30 pg/tube. The intra- and inter-assay coefficients of variation were 9.0% and 14.5%, respectively.

2.8. Pregnenolone ELISA

The pregnenolone concentration in the medium was determined by using an ELISA as previously described (Wang et al., 2011). Highly purified pregnenolone (Sigma Chemical Co.) was used as a standard. The standard or samples were combined with primary antibody (diluted 1:12,800 in blocking buffer), added to pregnenolone-BSA-coated 96-wells, and incubated at 37°C for 1 h. Following incubation, the wells were washed and incubated with a conjugated secondary antibody (IgG-HRP, diluted 1:5,000 in blocking buffer) at 37°C for 30 min. After washing, 3,3′,5,5′-tetramethylbenzidine (Sigma Chemical Co.) was added to the wells, and the plate was incubated at room temperature for 5 min under dark conditions. The reaction was terminated with 2 M HCl, and the absorbance was measured at 450 nm.

2.9. Statistical analysis

The results are expressed as the means ± S.E.M. A one-way analysis of variance (ANOVA, Steel and Torrie, 1960) was performed to determine body weight differences between the groups. All the other values were obtained from the NP and Veh groups, which were compared by *t*-test analysis. A P value of < 0.05 was considered to be statistically significant.

3. Results

First generation and second generation male offspring

3.1. Body weight and plasma levels of steroid hormones

As indicated by measurements taken every 3–4 days beginning at 3 weeks of age, animal body weights increased significantly in F_1 offspring exposed to NP *in utero* and during neonatal development (F_1 NP group) in comparison to the control animals (F_1 Veh group; $p < 0.05$, Figure 1A). In contrast, the body weights were not significantly different in the F_2 offspring (Figure 1B). The plasma ACTH, corticosterone, and aldosterone concentrations also significantly increased by 39%, 62%, and 55%, respectively, in the F_1 NP group in comparison to the F_1 Veh group ($p < 0.05$, Table 1). There were no changes in the plasma ACTH, corticosterone, and aldosterone concentrations of F_2 NP offspring relative to the control animals (Table 1). There was no difference in the prolactin level between experimental groups in F_1 and F_2 offspring (Table 1).

3.2. Liver

The 11β-HSD1 and GR levels in the liver were determined by western blot analysis (Figure 2A, B). The 11β-HSD1-to-β-actin protein ratio increased by approximately 15.9% in the F_1 NP group in comparison to the F_1 Veh group ($p < 0.05$, Figure 2A), but this effect was not observed in F_2 NP offspring (Figure 2B). In contrast, the GR-to-β-actin protein ratio increased moderately but not significantly in F_1 offspring (Figure 2A). The NP exposure effect on gestation and lactation was examined by measuring the NADPH-dependent oxidoreductase activity of

11β-HSD1 in the liver from F_1 adult offspring. A significant increase (30.1%) in 11β-HSD1 activity was observed in F_1 NP offspring ($p < 0.05$, Table 2), but not in F_2 NP offspring (Table 2), in comparison to the corresponding Veh groups.

3.3. Adipose tissue

The adipose tissue PPARγ level was determined by western blot analysis (Figure 3A, B). The PPARγ-to-GAPDH protein ratio was similar in the F_1 (Figure 3A) and F_2 (Figure 3B) offspring. In addition, there was no difference in 11β-HSD1 activity from F_1 (Table 2) and F_2 (Table 2) adult offspring. In contrast, the corticosterone concentration increased significantly in adipose tissue from F_1 NP offspring when compared to F_1 Veh offspring ($p < 0.05$, Table 2). There was no difference in the corticosterone concentration of F_2 offspring (Table 2).

3.4. Adrenal gland

StAR and GR adrenal gland levels were determined by western blot analysis (Figure 4A, B). The ratio of StAR-to-β-actin and GR-to-β-actin increased by approximately 14.7% and 23.2%, respectively, in the F_1 NP group ($p < 0.05$, Figure 4A), but not in the F_2 NP group (Figure 4B) when compared with the control. Moreover, a significant increase in 11β-hydroxylase activity (32.3%) in the adrenal cortex ($p < 0.05$, Table 2) and aldosterone synthase activity (38.6%) in the adrenal capsule ($p < 0.05$, Table 2) was observed in F_1 NP offspring, but not in F_2 NP offspring (Table 2) relative to the control. A significant increase in P450scc activity in the adrenal cortex of F_1 NP rat offspring was also observed ($p < 0.01$, Table 2), but not in F_2 NP offspring (Table 2) relative to the control.

4. Discussion

NP is an environmental toxicant that contaminates the food chain (Guenther et al., 2002; Raecker et al., 2011). It has been detected in human cord blood (Chen et al., 2008) and milk (Ademollo et al., 2008; Chen et al., 2010). Human NP exposure is apparently not limited only to the adult stage, but also affects the sensitive development period. For instance, adults living in Germany are exposed to NP levels of approximately 7.5 μg/day (Guenther et al., 2002), and infants are exposed to NP levels of 0.2−1.4 μg/day (Raecker et al., 2011). The environmental relative daily exposure dose is likely to be approximately a microgram.

In this study, hyperadrenalism was observed in F_1 offspring in the form of elevated plasma ACTH, corticosterone, and aldosterone levels, and by a decrease in hypothalamic-pituitary-adrenal (HPA) negative feedback system sensitivity. These observations correspond to an activated HPA axis by prenatal stress or glucocorticoid exposure during pregnancy (Cottrell and Seckl, 2009; Reynolds, 2013). Elevated 11β-HSD1 activity is associated with obesity and metabolic syndrome (Rask et al., 2001; Reynolds, 2013; Wake et al., 2003), and the increased liver 11β-HSD1 protein and enzymatic activity in our study is in agreement with previously published results. An elevation in the GR liver level is also indicative of hyperadrenalism. Moreover, the StAR and glucocorticoids levels were significantly increased in the adrenal gland. It is evident that NP exposure resulted in hyperadrenalism and weight increase in F_1 generation. For F_2 generation, there was no significant difference between NP and control groups. The F_2 generation status seems to have been set back to the 'default' stage (the same as the control). These observations support the hypothesis that epigenetic modifications by NP exposure can be reset.

Prolactin can enhance adrenal cell responses to ACTH stimulation (Jaroenporn et al., 2007a) and stimulate corticosterone release from adrenal cells (Jaroenporn et al., 2007b). Hyperprolactinemia is also associated with weight gain and loss (Galluzzi et al., 2005; Greenman et al., 1998). In this study, NP did not affect serum prolactin levels. These results are consistent with a study by Masutomi et al. (2004), who examined prolactin-positive cells in the pituitary gland following EDC exposure. Thus, the increase in F_1 offspring body weight is not likely to be mediated by an elevated prolactin level.

PPARγ is a key regulator of adipogenesis, and it is the target of some obesogens such as tributyltin and phthalates (Janesick and Blumberg, 2011, 2012). Hao et al. (2012) showed NP could induce PPARγ expression in the 3T3-L1 peradipocyte cell line, and mouse injection with NP increased PPARγ expression by 24 h. In our study, there was no significant difference in PPARγ levels between the control and treatment groups in both F_1 and F_2 offspring. Our data suggest that NP does not mediate PPARγ adipogenesis action.

With the exception of mutations, the DNA sequence in mammal cells and their zygotes is identical. Epigenetic modifications must take place for cells to differentiate into different types (Reik, 2007). A differentiated cell type normally remains in the same type. This trend means that epigenetic modification is stable enough to be passed along generations of cell cleavage and propagation. Epigenetic modifications are stable cellular events that are passed along generations, and they involve histone tail methylation/acetylation (Margueron et al., 2005), methylation at the fifth carbon of the cytosine base (CpG methylation) (Chen et al., 2005), non-coding RNA (Wall and Shi, 2003), and high-order chromatin organization (Wallace and Orr-Weaver, 2005). The underlying reasons why an epigenetic marker can be stably passed (inherited) through cell divisions would provide very

interesting future study subjects. The best studied epigenetic modification is cytosine base methylation and maintenance (Bostick et al., 2007; Sharif et al., 2007). The main machinery of this modification is nuclear protein 95 (Np95) or UHRF1. Np95 recognizes hemi-methylated DNA at replication sites and recruits DNA methyltransferase 1 (Dnmt1). Dnmt1 copies the methylation mark on the parental strand and delivers it to the daughter strand, thereby maintaining the epigenetic mark throughout cell division. Once the epigenetic mark enters into the somatic cell line, it has a lifelong and stable differentiation. The key concept is 'life long'; this term means the obesogen epigenetic modification effects last for the whole life span of the affected individual. This life long duration predisposes an individual to obesity may explain the limited success of weight control plans (Katan, 2009; Odermatt and Gumy, 2008; Stevens et al., 2012).

Now the question is how environmental pollutants impact upon epigenetic modification? The evolutionary process involves variation within populations and environmental selection. Because epigenetic modifications are stably maintained within somatic cells, the sensible expectation is to create variation before stabilization of epigenetic modifications during the developmental period. During this time, there must be a flexible period in which the organism responds to environmental factors. As a logical consideration, there is a plasticity period during embryonic/fetal development (Gluckman et al., 2007; Godfrey et al., 2010; Hanson et al., 2011). Developmental plasticity is a property in which different phenotypes are derived from a single set of genotypes in response to different developmental environments. A species has an evolutionary advantage if the next generation is better prepared for future environments. If the preparation matches the coming environment, both the species and individual will flourish. If the preparation mismatches the coming environment, then there must be a consequence. For reasons of species survival, evolution is more likely to select traits that have less impact on reproduction. A logical solution is to reproduce early in adult life and let the consequences gradually appear and become worse later in life (Morris et al., 2009). This concept is in agreement with the 'fetal origins of adult diseases' theory, which states that cues received from the maternal environment can modify the developmental trajectory (Barker et al., 2002; Gluckman and Hanson, 2004). The metabolic trajectory shift may be small at an early age, but the gap becomes larger later in life. Examples are undernourishment (Morris et al., 2009; Osmond and Barker, 2000) or stressed/glucocorticoid exposure (Cottrell and Seckl, 2009; Reynolds, 2013) babies are underweight and predisposed to obesity and metabolic syndrome later in life. Developmental exposure to obesogens apparently follows a similar pattern in human and animal models. In brief, developmental exposure to environmental factors results in epigenetic modifications (Skinner et al., 2010a, 2011). Epigenetic modifications shift the metabolic trajectory, predisposing individuals to obesity and metabolic syndrome later in life. This concept is supported by our data, which showed body weight gradually increasing in F_1 offspring after puberty.

There must be a way to reset those epigenetic modifications, or the accumulation of all evolutionarily disadvantage traits could finish the species. Although epigenetic marks maintain life long stability, they are erased and rebuilt in the transition to offspring. There are two periods during which genetic information undergoes genome-wide demethylation and re-methylation (Li, 2002; Morgan et al., 2005; Reik et al., 2001; Seisenberger et al., 2013). The first period occurs during early embryogenesis (i.e., before embryo implantation), and the second period occurs during gametogenesis (i.e., after implantation but before delivery). Beginning at the zygote stage, sperm DNA goes through active genome-wide demethylation while egg DNA goes through passive demethylation. These reprogramming processes seem to remove barriers and differentiation markers to restore the developmental potential of embryonic blastomeres. The global methylation pattern is subsequently restored to commit to a certain differentiation fate (Li, 2002; Morgan et al., 2005; Reik et al., 2001; Seisenberger et al., 2013). During gametogenesis, the inherited epigenetic signature (i.e., DNA methylation) of primordial germ cells is erased by another wave of remodeling, which restores developmental potency and gamete imprinting (Li, 2002; Morgan et al., 2005; Reik et al., 2001, 2007; Seisenberger et al., 2013). These epigenetic barriers will be broken and built once more at the zygote stage of the next generation. Thus, epigenetic markers caused by EDCs can be erased during early embryogenesis and avoided altogether if pregnant females are not exposed to EDCs again. The predisposition towards obesity and metabolic syndrome in adults can be alleviated in subsequent generations that were unaffected by NP. In theory, F_0 generational exposure to obesogens leads to F_1 generation adult obesity and metabolic syndrome, and F_2 generation males can return to normal endocrine function and should encounter less resistance in the control of obesity and metabolic syndrome. For females, NP exposure during the F_0 generation not only affects the F_1 generation at the embryo/fetus stage, but also affects the germ line in the fetus (F_2 generation) (Skinner, 2010b). It may take one more generation to observe recovery, which will be explored in the next study.

The hypothesis of this study is that epigenetic modifications can be re-set to a 'default' stage during a genome-wide breaking and building process. The data in this study support this hypothesis. In the study model, it only takes one generation (F_0 treated→F_1 affected→F_2 recovery) to recover from the predisposition to obesity and metabolic syndrome. One generation for recovery may not be universally applicable. Variations in the toxicity of environmental chemicals, the timing and duration of exposure, toxicant concentrations, and effect mechanism,

i.e. somatic or germline imprinting, will alter the epigenetic modification status. At somatic level, it may take more than 1 set of cycles (embryogenesis and gametogenesis) to erase and rebuild the 'default' status. It is not clear about the reset of germline imprinting issue. The key concepts from this study are epigenetic modification gives life long impact and epigenetic modification can be erased and rebuilt through generation(s) pass on. The exploitations of the study conclusion are there is still hope. The current issues are how to prepare for the coming and mounting societal healthcare burdern i.e. metabolic syndrome and obesity as a disease, and how to avoid serial generational exposure, i.e. environmental protection.

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Tables and Figures

	Vehicle Group	NP Group	p value
1st generation (n=11-18)			
ACTH (ng/ml)	0.407±0.037	0.565±0.076	$p < 0.05$
prolactin (ng/ml)	16.011±1.725	18.322±1.497	$p > 0.05$
corticosterone (ng/ml)	66.75±14.39	107.99±18.90	$p < 0.05$
aldosterone (ng/ml)	0.163±0.038	0.252±0.031	$p < 0.05$
2nd generation (n=12-18)			
ACTH (ng/ml)	0.260±0.030	0.259±0.030	$p > 0.05$
prolactin (ng/ml)	14.469±1.352	13.945±1.183	$p > 0.05$
corticosterone (ng/ml)	74.61±16.93	91.73±18.62	$p > 0.05$
aldosterone (ng/ml)	0.281±0.042	0.274±0.053	$p > 0.05$

Table 1. The concentration of ACTH, prolactin, corticosterone and aldosterone in plasma of F_1 and F_2 offspring. The concentrations are measured by RIA. Values are shown as mean ± S.M.E. p value was compared with vehicle group in the same generation.

	Vehicle Group	NP Group	p value
1st generation (n=14-18)			
11 -HSD1 activity in liver (ng/h/g protein)	1067.23±114.47	1388.88±84.46	$p < 0.05$
11 -HSD1 activity in adipose (ng/h/ g protein)	1822.75±46.75	1792.34±82.34	$p > 0.05$
P450scc activity (ng/h/ g protein)	0.0587±0.0067	0.1194±0.0158	$p < 0.01$
11 -hydroxylase activity (ng/h/ g protein)	0.291±0.032	0.385±0.037	$p < 0.05$
Aldosterone synthase activity (ng/h/ g protein)	0.250±0.027	0.346±0.036	$p < 0.05$
corticosterone content in adipose (ng/g adipose)	16.41±2.42	23.30±2.99	$p < 0.05$
2nd generation (n=11-18)			
11 -HSD1 activity in liver (ng/h/ g protein)	1929.28±99.94	1761.07±73.42	$p > 0.05$
11 -HSD1 activity in adipose (ng/h/ g protein)	906.34±114.61	926.26±130.01	$p > 0.05$
P450scc activity (ng/h/ g protein)	0.0708±0.0127	0.0482±0.0093	$p > 0.05$
11 -hydroxylase activity (ng/h/ g protein)	0.370±0.074	0.243±0.037	$p > 0.05$
aldosterone synthase activity (ng/h/ g protein)	0.284±0.055	0.301±0.080	$p > 0.05$
corticosterone content in adipose (ng/g adipose)	16.65±1.37	20.76±2.02	$p > 0.05$

Table 2 The enzyme activity or adipose corticosterone content for F_1 and F2 offspring. Values are shown as mean ± S.M.E. p value was compared with vehicle group of the same generation.

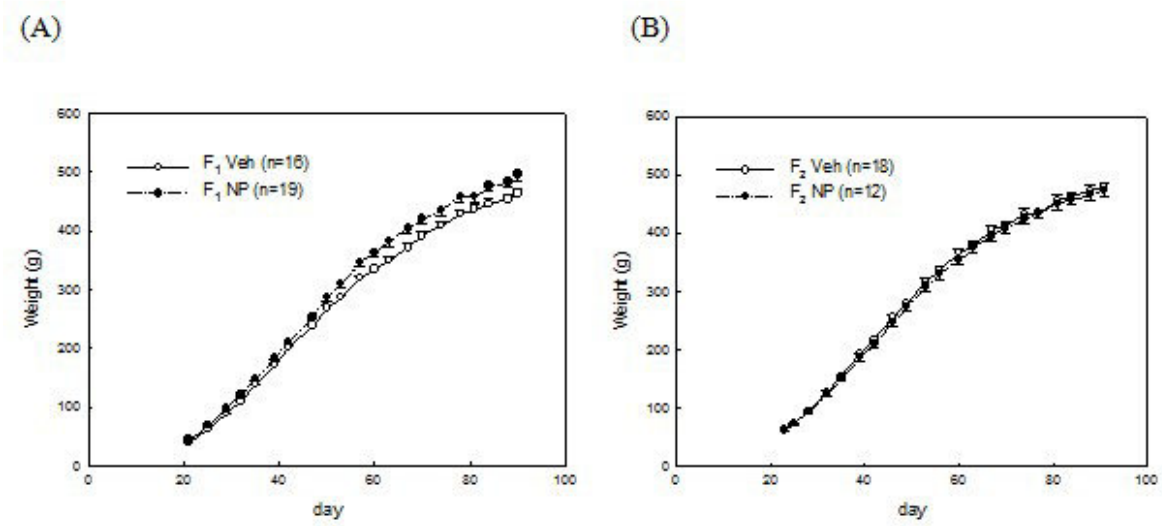
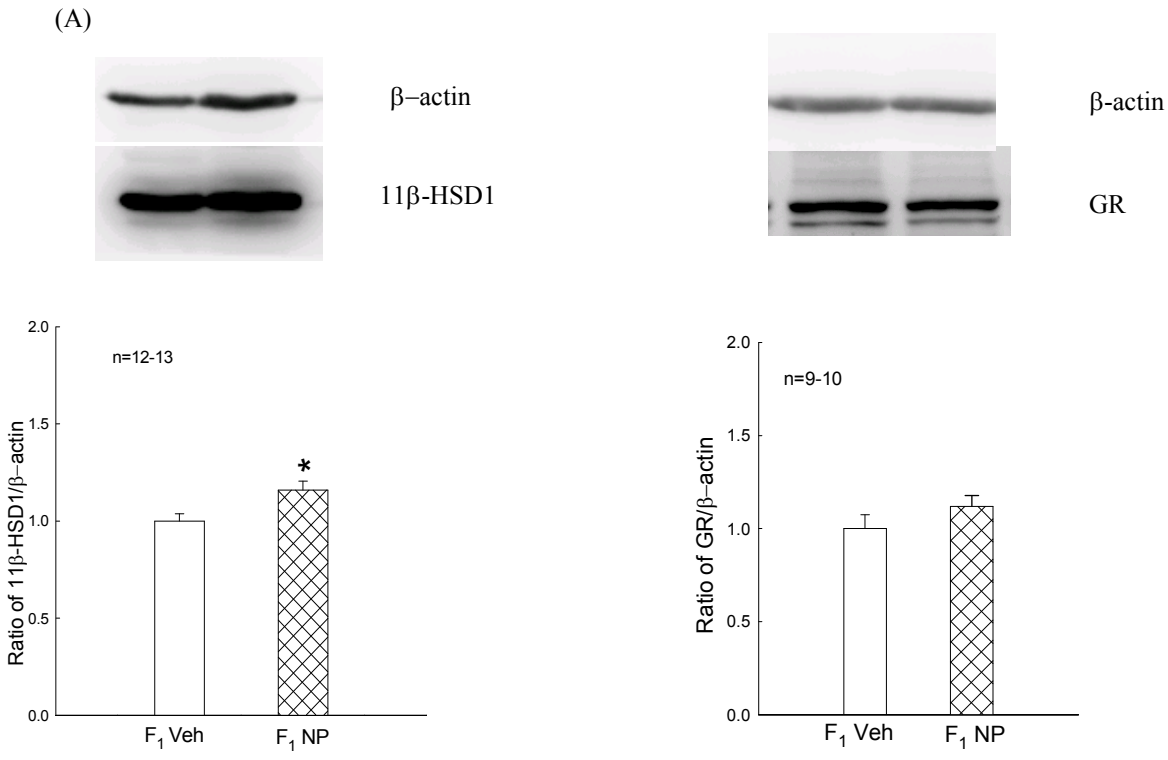


Figure 1. Body weights of F_1 and F_2 males. (A) Body weights of F_1 NP males compared with F_1 Veh males. (B) Body weights of F_2 NP males compared with F_2 Veh males. The values are presented as the means ±S.E.M.



(B)

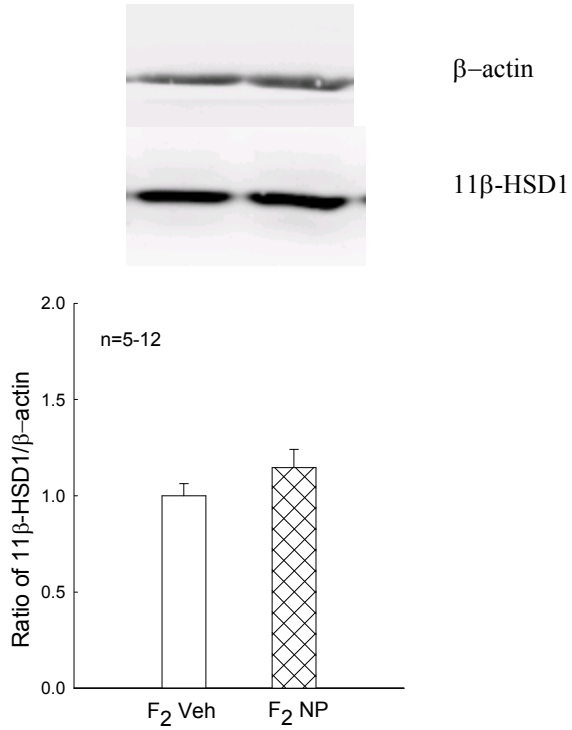


Figure 2. 11β- SD1 (32 kDa) and/or GR (94 kDa) levels in the liver from F_1 (A) and F_2 (B) NP and Veh males were determined by western blot analysis, and the values were normalized against β-actin. * $p < 0.05$ compared with vehicle group of the same generation.

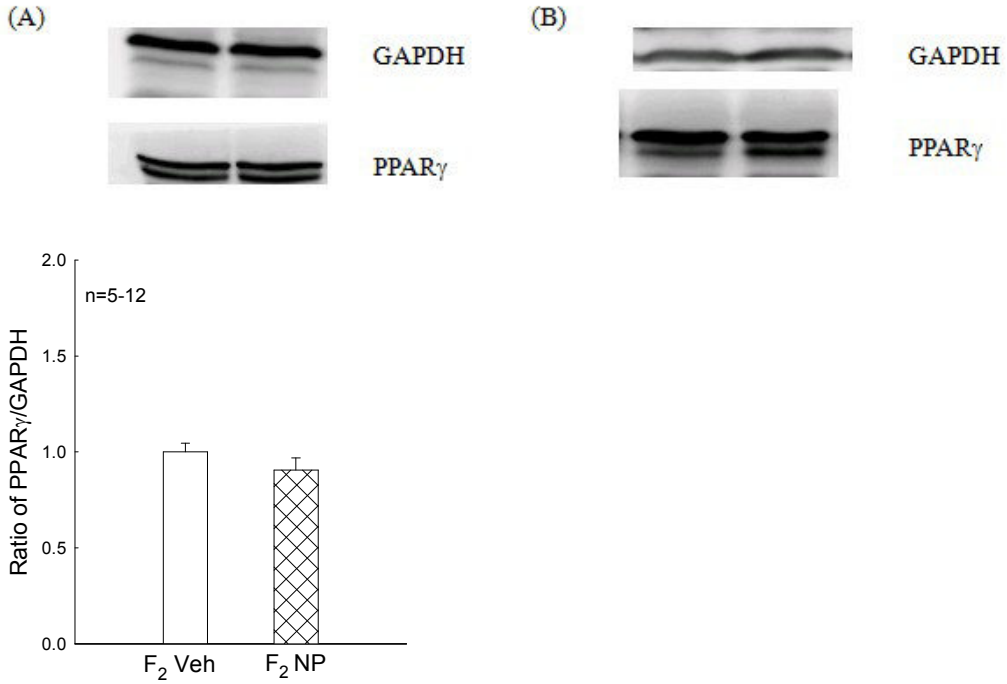


Figure 3. The PPARγ (57 kDa) level in adipose tissue from F_1 (A) and F_2 (B) NP and Veh males was determined by western blot analysis, and the values were normalized against GAPDH.

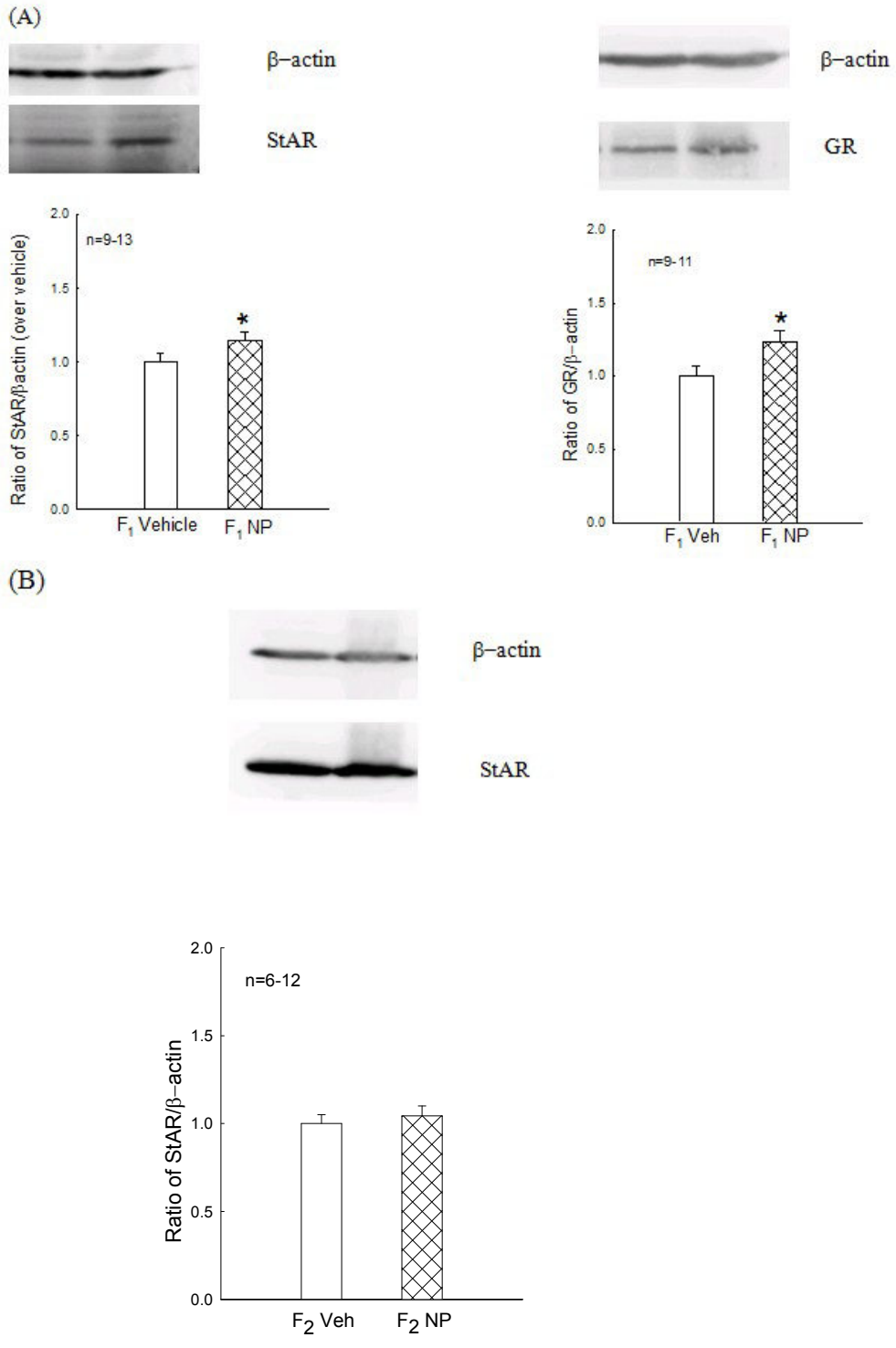


Figure 4. StAR (30 kDa) and/or GR (94 kDa) levels in the adrenal gland from F_1 (A) and F_2 (B) NP and Veh males were determined by western blot analysis, and the values were normalized against β-actin. *: $p < 0.05$ compared with vehicle group of the same generation.

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