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Developmental Origins of Health and Disease

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Review Article

Fetal Programming of Body Composition, Obesity, and Metabolic Function: The Role of Intrauterine Stress and Stress Biology

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Epidemiological, clinical, physiological, cellular, and molecular evidence suggests that the origins of obesity and metabolic dysfunction can be traced back to intrauterine life and supports an important role for maternal nutrition prior to and during gestation in fetal programming. The elucidation of underlying mechanisms is an area of interest and intense investigation. In this perspectives paper we propose that in addition to maternal nutrition-related processes it may be important to concurrently consider the potential role of intrauterine stress and stress biology. We frame our arguments in the larger context of an evolutionary-developmental perspective that supports roles for both nutrition and stress as key environmental conditions driving natural selection and developmental plasticity. We suggest that intrauterine stress exposure may interact with the nutritional milieu, and that stress biology may represent an underlying mechanism mediating the effects of diverse intrauterine perturbations, including but not limited to maternal nutritional insults (undernutrition and overnutrition), on brain and peripheral targets of programming of body composition, energy balance homeostasis, and metabolic function. We discuss putative maternal-placental-fetal endocrine and immune/inflammatory candidate mechanisms that may underlie the long-term effects of intrauterine stress. We conclude with a commentary of the implications for future research and clinical practice.

1. Introduction

A growing body of empirical evidence suggests that the origins of obesity and metabolic dysfunction can be traced back to the intrauterine period of life, at which time the developing fetus is acted upon by and responds to suboptimal conditions during critical periods of cellular proliferation, differentiation, and maturation by producing structural and functional changes in cells, tissues and organ systems. These changes, in turn, may have long-term consequences to increase the individual's risk for developing a range of complex common disorders including, but not limited to, obesity and metabolic dysfunction (i.e., the concept of fetal programming of health and disease risk [1]). A large number of human and animal studies of fetal programming of obesity and metabolic dysfunction have focused on the critical role of maternal nutrition prior to or during gestation and have produced important findings and insights (reviewed in [2–5]). Questions currently under investigation in this context include those related to mechanisms or pathways by which nutritional programming can exert life-long effects on the developing organism. Some of the major nutrition-related pathways discussed in the current literature relate to the effects of maternal nutritional insults on

maternal-placental-fetal glucose/insulin physiology and their downstream effects on the developing brain and peripheral systems in the fetal compartment. In this perspectives paper, we argue that it may be important to also simultaneously consider the potential role of intrauterine stress and stress biology for the following reasons: (a) from an evolutionary-developmental perspective, energy availability (i.e., nutrition) and challenges that have the potential to impact the structural or functional integrity and survival of the organism (i.e., stress) represent the most important environmental conditions underlying natural selection and developmental plasticity along all time scales. It is therefore likely and plausible that stress represents an important aspect of the intrauterine environment that would be expected to influence many, if not all, developmental outcomes. (b) stress-related biological factors may exert direct effects on fetal targets of programming of body composition and metabolic function. (c) many of the effects of nutritional insults (both undernutrition and overnutrition) may be mediated by common stress-related pathways involving the hypothalamic-pituitary-adrenal (HPA) axis and its end product, cortisol. Hence, stress biology may represent a common underlying mechanism. (d) stress and stress-related biological processes are known to alter nutrition at several levels, including caloric intake, selection of food types, and metabolic fate of energy. Conversely, nutritional status is also known to alter stress at multiple levels in the brain and periphery, including appraisals of potentially stressful circumstances, psychological and physiological stress responses, and feedback regulation. Hence, in natural settings it is likely that the effects of either nutrition or stress are modified by or conditioned upon the state of the other. In other words, interaction effects, and not main effects, are the more likely scenario underlying causation in the context of complex common disorders including obesity and metabolic function. This issue is particularly important in the human context since nutritional insults and stress tend to cooccur in populations across the world.

For these reasons, we highlight and review below the effects of stress and stress biology on fetal programming of body composition, obesity, and metabolic function. We review empirical evidence for interactive effects between stress and nutrition, describe findings from some of our own recent studies on prenatal stress and stress biology, and discuss putative maternal-placental-fetal endocrine and immune/inflammatory candidate mechanisms that may underlie and mediate short- and long-term effects of prenatal stress on the developing human embryo and fetus, with a specific focus on body composition, metabolic function, and obesity risk. We conclude with a commentary of the implications for research and clinical practice.

2. Rationale for Considering a Role for Stress in Fetal Programming

The origins of health and disease susceptibility for many of the complex, common disorders that confer the major, global burden of disease in developed societies as well as other societies in rapid transition can be traced back to the intrauterine period of life. Development is a plastic process, wherein a range of different phenotypes can be expressed from a given genotype (contained within the fertilized zygote). The unfolding of all developmental processes across the multicontoured landscape from genotype to phenotype is context-dependent, wherein the developing embryo/fetus responds to, or is acted upon by, conditions in the internal or external environment during sensitive periods of cellular proliferation, differentiation and maturation, resulting in structural and functional changes in cells, tissues, and organ systems. These changes may, in turn, either independently or through interactions with subsequent developmental processes and environments, have short- and/or long-term consequences for health and disease susceptibility. These concepts have variously been referred to as the fetal or developmental origins of health and disease risk [1, 6].

The rationale for considering a role for stress and stress biology in fetal programming of child and adult obesity and metabolic dysfunction derives, in part, from concepts in evolutionary biology and developmental plasticity. From conception onwards the mother and her developing fetus both play an obligatory, active role in all aspects of development. Based on the consideration that key environmental conditions that have shaped evolutionary selection and developmental plasticity include not only variation in energy substrate availability (i.e., nutrition) but also challenges that have the potential to impact the structural or functional integrity and survival of the organism (i.e., stress), it is likely and plausible that prenatal stress represents an important aspect of the intrauterine environment that would be expected to influence many, if not all, developmental outcomes [7]. Moreover, we submit the application of a prenatal stress and stress biology framework offers an excellent model system for the study of intrauterine development and associated developmental, birth and subsequent health-related phenotypes because it is increasingly apparent that the developing fetus acquires and incorporates information about the nature of its environment in part via the same biological systems that in an already-developed individual mediate adaptation and central and peripheral responses to endogenous and exogenous stress (i.e., the maternal-placental-fetal neuroendocrine and immune systems [8]).

Another compelling rationale for considering a role for *in utero* stress as a contributor to subsequent risk of obesity and metabolic dysfunction derives from the effort to elucidate and better understand the underlying reason(s) for the well-documented, persistent and large socioeconomic and racial/ethnic disparities in the population distribution of these outcomes in the US and other developed nations. Many of the factors that disproportionally affect socially disadvantaged individuals, such as medical care, diet/nutrition, and health-related behaviors, have been shown to play only a limited role in accounting for these disparities [9–12]. The search for alternate explanations has led to the hypothesis that high levels of stress may, in part, independently, or in combination with other factors, account for these disparities, because the experience of social disadvantage and minority racial/ethnic status is

characterized by higher levels of psychosocial stress and lack of resources, and because stress and stress-related biological processes have been implicated in a wide array of adverse reproductive, developmental, and other health outcomes [9, 13].

A large body of the literature supports the notion that conditions in the early postnatal period of life (e.g., behavioral or nutritional stress) can induce changes in the metabolic, endocrine, cardiovascular, and behavioral phenotypes, and these effects could be independent of prenatal exposures or could moderate or mediate the effects of prenatal adversity. Several of these findings are derived from animal (rodent) models, in which maturational status over the first two weeks of postnatal life is approximately equivalent to that of the human during the third trimester of gestation. A comprehensive summary of the effects of early postnatal life stress is beyond the scope of the current paper, but we refer to reader to excellent recent reviews on this topic [14, 15].

3. The Role of Context: Potential Interactive Effects between Stress and Nutrition

Obesity and metabolic dysfunction are complex, multifactorial outcomes. At the individual level, the major risk categories include sociodemographic, nutritional, historical, biophysical, obstetric, behavioral, psychosocial, genetic, and other environmental factors. Studies of the effects of stress and stress-related processes on these outcomes generally treat other risk factors as potential confounding variables and attempt to account (adjust) for their putative effects by either study design (subject selection criteria) or statistical adjustment. However, emerging concepts of causation for complex common disorders, including but not limited to obesity and metabolic dysfunction, suggest it is not only possible, but in fact probable, that causation does not reside in any single factor or in the additive effects of numerous factors, but lies at the interface between multiple risk factors (interaction or multiplicative [16]). We consider here, by way of example and illustration of this critically important concept, the potential interactive effects between stress and nutrition.

As discussed briefly in the preceding section, the two fundamental processes that are believed to shape evolutionary selection and developmental plasticity are variation in energy substrate availability (nutrition) and challenges that have the potential to impact the structural or functional integrity and survival of the organism (stress). Maternal nutrition, assessed by indicators of body size such as body mass index (BMI), nutritional intake or serum measures of nutritional biomarkers, is a well-established risk factor for childhood and adult obesity and metabolic dysfunction. Growing evidence supports the concept of a bidirectional interaction between nutrition and stress, such that the effects of nutrition on health may vary as a function of stress, or that the effects of stress on health may vary as a function of nutritional status. For example, several experimental studies in animals have demonstrated that nutritional manipulations, particularly in the preconception or early pregnancy period, may produce their effects on maternal and fetal outcomes via alterations in stress biology (cortisol, inflammatory cytokines [17–25]). Conversely, studies in animals and humans of stress induction (by exposure to laboratory-based stressors or endocrine stress analogues) have demonstrated effects on feeding behavior, food choice (high-calorie dense food preference) and the metabolic fate of food in target tissues [26–30]. For example, chronic stress or cortisol administration motivates people to select high-fat food and to overeat [26, 29], and corticotrophin-releasing hormone (CRH) infusion in healthy human adults also increases subsequent food intake [28]. Furthermore, chronic stress has the potential to impair sleep, and short sleep duration is a predictor of weight gain [31]. In addition, cortisol increases insulin levels [32, 33]. Although insulin is anabolic and under normal basal conditions can increase both lean and fat mass, coelevation of insulin with cortisol preferentially increases abdominal fat stores [34, 35]. Further evidence of an interaction between stress and nutrition comes from a recent study in humans demonstrating that under conditions of stress the brain's energy need increases, and it actively "demands" energy from the periphery (a concept termed "brain-pull," [36]). It is hypothesized that under conditions of high energy demand the brain can activate its stress systems, that is, the sympathetic nervous system (SNS) and the hypothalamus pituitary adrenal (HPA) axis. Once stress networks in the upper brain stem including the ventromedial hypothalamus (VMH) and the paraventricular nucleus (PVN) are activated, energy—particularly glucose—is allocated to the brain. With SNS activation, insulin secretion from the beta cells is suppressed, and the insulin-dependent glucose uptake via GLUT4 into the body periphery becomes limited, referred to as "cerebral insulin suppression" (CIS). As a consequence of CIS, glucose is available via insulin-independent GLUT1-transport across the blood-brain barrier. This hypothesis was tested using an experimental design, wherein healthy young adults underwent a laboratory stress test and a control session. Acute stress exposure increased carbohydrate intake from a rich buffet compared to the control session. While these stress-extra carbohydrates increased blood glucose concentrations, they did not increase serum insulin concentrations. The ability to suppress insulin secretion was found to be linked to the sympathoadrenal stress response [27]. The authors speculated that disturbances of this "brain-pull" mechanism may be related to the onset of obesity, because in the case of incompetent "brain-pull" food intake has to be increased in order to ensure the brain's energy supply under conditions of stress [36].

We note that only a small number of studies have examined the relationship between maternal stress and diet or nutritional state in pregnancy. A study by Hurley et al. [37] found that pregnant women who were more fatigued, stressed, and anxious in mid-pregnancy consumed more food (increased macronutrient intake) but concurrently decreased their intake of some micronutrients. Another recent study demonstrated that the level of maternal stress during pregnancy was positively associated with prepregnancy BMI [38]. In an animal model, the interactive effects

of maternal stress and nutrition on the subsequent risk of offspring obesity were investigated [24]. Pregnant rats were maintained on standard or high-fat diet throughout gestation and lactation. Offspring from dams that experienced prenatal stress and/or were on a high-fat diet weighed more beginning on postnatal day 7 compared to standard control pups. Access to high-fat diet at weaning increased the body weight effect through early adulthood and was attributable to greater adiposity. Furthermore, pups weaned on to a high-fat diet had impaired glucose tolerance if their dams were on a high-fat diet, experienced prenatal stress, or both [24].

Findings from a recent study on maternal high-fat diet during pregnancy suggest that the effects on offspring hypertension in adult life are mediated through an exacerbated sympathetic tone that arises very early in life [39]. We note that increased sympathetic tone is also associated with alterations in the stress response.

Despite the plausibility of stress-nutrition interaction effects in the context of pregnancy, we are not aware of any human studies to date that have examined these interactive effects during pregnancy on offspring body composition and metabolic function.

4. Stress-Related Maternal-Placental-Fetal Endocrine and Immune Processes as Potential Mediators of Fetal Programming of Health and Disease

The fetal programming hypothesis has led to the search for underlying mechanisms by which disparate intrauterine insults exert a multitude of effects on different physiological systems in the developing offspring. A question of particular interest relates to whether these biological mechanisms are exposure and/or outcome-specific, or whether there may be some common mechanisms that mediate the effects of various exposures on a range of disparate outcomes. We suggest that stress-related maternal-placental-fetal endocrine and immune processes in gestation constitute an attractive underlying common candidate mechanism because they are responsive to many classes of intrauterine perturbations and they act on multiple targets of fetal programming [8, 40]. Unlike exposure to toxins and teratogens, it is important to appreciate the fact that maternal-placental-fetal hormones and cytokines play an essential and obligatory role in orchestrating key events underlying cellular growth, replication and differentiation in the brain and peripheral tissues [41–46]. Thus, perturbations in the level and/or time of exposure of these biologic effectors are likely to produce alterations of normal structure and function. It is also important to appreciate that the state of pregnancy itself produces major and progressive alterations in the function of these systems, and that these changes may have important implications for altering the responsivity of these systems to exogenous or endogenous perturbations and hence their downstream effects on fetal targets of programming.

4.1. Stress Biology in Human Pregnancy. Stress biology refers to the set of biological adaptations in response to challenges or demands that threaten or are perceived to have the potential to threaten the stability of the internal milieu of the organism. The nervous, endocrine, immune, and vascular systems play a major role in adaptations to stress. There are no direct neural, vascular, or other connections between the mother and her developing fetus—all communication between the maternal and fetal compartments is mediated via the placenta, an organ of fetal origin. Based on the physiology of stress, parturition and the evidence linking maternal stress to earlier delivery, we have previously proposed a biobehavioral framework of stress and adverse birth outcomes [8], that may also be applicable in the present context.

Pregnancy produces major alterations in neuroendocrine and immune function, including changes in hormone and cytokine levels and control mechanisms (feedback loops), that are crucial in providing a favorable environment within the uterus and fetal compartment for growth, differentiation and maturation, and conveying signals when the fetus is ready for extrauterine life. Starting very early in gestation the placenta, the first fetal organ to develop and function, produces hormones, neuropeptides, growth factors, and cytokines, and appears to function in a manner resembling that of compressed hypothalamic-pituitary-target systems [47].

4.1.1. Maternal-Placental-Fetal Stress-Related Endocrine Function. Glucocorticoid physiology (cortisol in humans) has received extensive and well-placed consideration as a critical endocrine mediator of fetal programming, with an emphasis on not only hormone production but also hormone action mediated by tissue-specific glucocorticoid receptor expression, sensitivity and affinity, and by maternal-fetal transfer mediated by the activity of the placental 11β-hydroxysteroid dehydrogenase enzyme system (see [48] for a recent review). Less well recognized is the potential and perhaps equally important role of the peptide corticotrophin-releasing hormone (CRH). In primates, but not other mammals, the placenta synthesizes and releases CRH in large amounts into the fetal and maternal circulations. In contrast to the inhibitory influence on hypothalamic CRH production, cortisol stimulates placental CRH production [49], and this positive feedback loop results in a progressive amplification of CRH and cortisol production over the course of gestation [50].

4.1.2. Maternal-Placental-Fetal Stress-Related Immune Function. With respect to the immune axis, a major endeavor of pregnancy-related alterations in immune function is to achieve and maintain the optimal balance between tolerating the fetal semiallograft while not suppressing maternal immune responses to an extent that increases maternal or fetal susceptibility to infection. Thus, a generalized reduction of maternal immune responsiveness occurs during pregnancy, mediated by hormonal changes (e.g., increased levels of progesterone), trophoblast expression of key immunomodulatory molecules, and a progressive switch from a TH_1/TH_2 balance to a predominantly T-helper 2-type pattern of cytokines [51].

4.1.3. Interactions between Maternal-Placental-Fetal Neuroendocrine, Immune-Inflammatory, and Vascular Pathways in Pregnancy. Although distinct neuroendocrine, immune/inflammatory, and vascular pathways have been described, growing evidence suggests that these and other physiological systems involved in pregnancy are highly interrelated, and that they extensively regulate and counterregulate one another. The potential complexity of the interrelationships among these physiologic systems is seen when considering the role of infection in the etiology of adverse fetal developmental and birth outcomes. For example, inflammatory cytokines that are produced in response to infection, such as TNF-α, IL-1β, and IL-6, can activate components of the MPF neuroendocrine system [52–54]. Conversely, it is also known that HPA hormones such as CRH and cortisol influence the production of cytokines and modulate the inflammatory response to infection [55–57]. Central CRH, acting via glucocorticoids and catecholamines, inhibits inflammation, whereas CRH directly secreted by peripheral nerves and mast cells stimulates local inflammation [58]. Impaired nutrient and oxygen exchange associated with uteroplacental vasculopathy may stress the fetus and result in increased production of placental-fetal hormones such as CRH, while placental CRH, in turn, may influence fetal-placental circulation [59]. Thus, the relationship between immune and endocrine alterations during pregnancy to adverse metabolic outcomes and increased risk of obesity on the offspring is likely to involve complex interactions between the endocrine, immune, and vascular systems.

4.2. Prenatal Stress and Maternal-Placental-Fetal Endocrine and Immune Function. Substantial evidence in nonpregnant humans and animals demonstrates that stress exposure produces activation of the neuroendocrine system (e.g., HPA axis) and exaggerated inflammatory responses [60, 61]; however, these associations cannot be assumed to also be present in the pregnant state because the above-described changes in endocrine and immune physiology have consequences for attenuating the responsivity of these systems to stress. With respect to prenatal psychosocial stress-related biological pathways, some of our earlier studies were among the first to demonstrate that despite the large pregnancy-associated changes in maternal endocrine physiology, the system is responsive to maternal psychosocial states (such as high stress and low social support) [62]. Our more recent studies on maternal stress responses in human pregnancy are among the first to demonstrate that maternal psychophysiological stress responses are progressively attenuated with advancing gestation [63], and that after accounting for the effects of other established risk factors, the degree of attenuation is a significant predictor of shortened length of gestation and earlier delivery [64].

Studies by other groups have reported that elevated psychosocial stress in pregnant women is associated with higher circulating levels of inflammatory markers like C-reactive protein (CRP) and the proinflammatory cytokines IL-1b, IL-6, and TNF-α, with lower circulating levels of the anti-inflammatory cytokine IL-10 and *ex vivo* endotoxin (LPS)-stimulated levels of IL-1b and IL-6 [65, 66]. Another recent study of pro-inflammatory responses to an *in vivo* antigen challenge (influenza virus vaccination) in pregnant women reported an association between depressive symptoms and sensitization of the inflammatory cytokine responses [66].

In addition to psychosocial stress, substantial *in vitro* and *in vivo* evidence indicates that maternal-placental-fetal endocrine and immune processes during pregnancy respond to a variety of other maternal and intrauterine perturbations, including biological effectors of stress [67–72], obstetric risk conditions such as preeclampsia, pregnancy-induced hypertension [57–70], gestational diabetes [73, 74], infection [75–78], reduced uteroplacental blood flow [79, 80], and behavioral factors such as the constituents of maternal diet, over- and under-nutrition, and smoking [17–23, 81, 82].

Based on these findings, it is apparent that measures of maternal-fetal endocrine and immune/inflammatory stress markers capture physiological responses to a wide range of intrauterine perturbations including, but not limited to prenatal stress. In accordance with our suggestion that stress-related maternal-placental-fetal endocrine and immune processes in gestation constitute an attractive candidate mechanism for fetal programming, a recent *JAMA* editorial [83] on an article reporting an increase in the prevalence of several categories of chronic illness in childhood, including obesity, asthma, and ADHD [84], speculates that there may be common early risks underlying these conditions that are triggering development of aberrant physiologic pathways. The editorial suggests that adverse early experiences that affect stress-sensitive physiologic systems (endocrine/metabolic, immune) may contribute to not only the onset of childhood illness but also predispose the same individuals to develop age-related diseases as adults.

5. Long-Term Effects of Prenatal Stress Exposure on Human Adult Physiology and Health

The majority of human epidemiologic studies of the fetal programming hypothesis have operationalized unfavorable intrauterine environments using indicators of adverse birth outcomes such as low birth weight. We and others have argued that the long-term effects on child or adult disease-related phenotypes of interest may not necessarily be mediated by adverse birth outcomes. For example, several experimental studies in animals suggest that maternal exposure to psychosocial stress during gestation can independently exert long-term effects on several central and peripheral systems in the offspring, and that titration of the prenatal stress exposure dose can produce significant long-term effects without necessarily altering the birth phenotype [85–89]. However, only a very small number of studies have investigated this issue in humans. As a first step to addressing this question, we conducted a study using a retrospective case-control design in a sample of healthy young adults born to mothers with healthy pregnancies and normal birth

outcomes. One half of the study population of young adults was born to mothers who had experienced a major stressful life event during the index pregnancy (prenatal stress group (PS)), whereas the other half was a sociodemographically matched population with no history of maternal exposure to prenatal stress (comparison group (CG)). We selected a study population of younger as opposed to older adults in order to focus on predisease markers of physiological dysregulation of metabolic, endocrine, and immune systems as early predictors of disease susceptibility. The potential effects of other established obstetric, newborn, and childhood risk factors on adult health were controlled using a stringent set of exclusionary criteria. Maternal and child medical records were obtained and screened to exclude presence of any maternal acute or chronic diseases, obstetric complications (e.g., gestational diabetes, hypertension/preeclampsia, and infection), unhealthy behaviors (smoking), adverse birth outcomes (preterm birth, low birth weight), newborn complications, and history of any major childhood or current diseases (obesity, diabetes, asthma, and adverse neurodevelopmental or psychiatric conditions). Study assessments were performed to quantify health and physiological markers of disease risk, including (i) body composition and glucose-insulin metabolism (BMI and percent fat mass; basal and postoral glucose tolerance test levels of glucose, insulin, leptin, adiponectin; fasting lipid profile), (ii) endocrine function (basal and post behavioral/pharmacological stress levels of pituitary-adrenal stress hormones, chronobiological regulation of adrenal function, and assessment of HPA-axis feedback sensitivity), (iii) immune function (immune cell trafficking and phytohemagglutinin (PHA)-stimulated production of pro- and anti-inflammatory and TH_1/TH_2 cytokines), (iv) cognitive function (working memory under basal and hydrocortisone conditions), and (v) cellular aging (as indexed by leukocyte telomere length). Because subtle physiological differences in disease susceptibility are often not detected in basal state, we employed appropriate challenge tests to quantify the function of these systems under stimulated conditions (e.g., oral glucose challenge, ACTH stimulation test, PHA-stimulated immune responses, and working memory after cortisol administration).

Our results (summarized in Table 1) indicated that the young adults exposed during intrauterine life to maternal psychosocial stress consistently exhibited significant dysregulation of all these key physiological parameters, thereby placing them at increased risk for developing complex common disorders. Specifically, individuals in the PS group exhibited higher BMI and percent body fat, primary insulin resistance, and a lipid profile consistent with the metabolic syndrome [90]; altered immune function with a TH2 shift in the TH1/TH2 balance (consistent with increased risk of asthma and autoimmune disorders [92]); altered endocrine function, with an increased ACTH and reduced cortisol levels during pharmacological and psychological stimulation paradigms (consistent with the high-risk endocrine profile exhibited by individuals exposed to early life abuse [95]); accelerated cellular aging (as indexed by shortened leukocyte telomere length that extrapolated to approximately a 3.5-year increase in the rate of cell aging [82]); and impaired prefrontal cortex (PFC)-related cognitive performance (impairments in working memory performance after hydrocortisone administration) [93]. Interestingly, stress-related changes in PFC function are believed to play a role in alterations of hypothalamic energy balance homeostasis circuits and obesity risk (see, for example, [96, 97]) thereby suggesting that prenatal stress may program brain regions that are associated with the control of energy intake. Consistent with the finding on cognitive function are results from one of our other recent prospective, longitudinal studies on the long-term effects of prenatal stress (anxiety) on child brain morphology. After excluding cases with low birth weight and adjusting for total gray matter volume, age, gestational age at birth, handedness, and postpartum stress, maternal pregnancy-specific anxiety in mid-gestation was associated with gray matter volume reductions in several child brain regions, including the prefrontal cortex [98].

Taken together, our findings suggest that *in utero* exposure to prenatal psychosocial stress may confer increased long-term risk of a range of negative physiological and cognitive health outcomes in humans; these effects are independent from those of other established obstetric and childhood risk factors; these long-term effects are not necessarily mediated by unfavorable birth outcomes. It is noteworthy that our above-described finding on body composition is consistent with a more recent report in a large, national cohort sample linking prepregnancy and prenatal stress exposure related to maternal bereavement to risk of childhood overweight [99], and our finding on immune function is consistent with another recent report linking prenatal maternal anxiety with infant illnesses and antibiotic use [100]. We also note that our findings on prenatal stress-associated altered immune, endocrine, cell aging, and cognitive function all converge in a manner consistent with the programming of body composition, obesity risk, and metabolic dysfunction.

6. Fetal Programming of Body Composition, Metabolic Function, and Obesity Risk

Continuing with the theme of a common underlying biological mechanism, in this section we address the issue of the potential impact of intrauterine stress biology on multiple targets of fetal programming related to body composition, metabolic function and obesity risk (see also [40]).

Obesity (or, to be more precise, adiposity) is recognized as one of the most serious health problems in the US and other societies. At the individual level, obesity results when energy intake exceeds energy expenditure. However, the relationship between excess energy intake and adiposity is not linear and monotonic; there is wide variation among children or adults at identical levels of excess energy intake in their propensity to gain weight and accrue fat mass. This variation across individuals defines susceptibility for developing obesity/adiposity. Once an individual becomes obese, it is difficult to lose weight, and even more difficult to sustain weight loss, because of the remarkable efficiency of energy balance homeostasis mechanisms [101–103]. For

Table 1: Long-term effects of prenatal stress exposure in young adults: summary of our studies.

Outcome	Finding	Potential implications	Reference
Body composition and metabolic function	↑ BMI; ↑ % body fat ↑ Insulin 2 h after oral glucose tolerance test ↑ Leptin ↓ Fasting HDL; ↑ fasting VLDL	Risk for cardiometabolic disorders/type 2 diabetes	Entringer et al. 2008 *Am J Ob Gyn* [90]
Endocrine system	↑ ACTH, ↓ cortisol in response to psychosocial stress test ↓ Cortisol levels in response to $ACTH_{1-24}$ stimulation test	Susceptibility for psychosomatic disorders	Entringer et al. 2009 *Horm Behav* [91]
Immune system	TH2 shift in TH1/TH2 balance after PHA stimulation ↑ IL-6, IL-10 after PHA stimulation	Risk for allergies, atopic disease, and asthma	Entringer et al. 2008 *Dev Psychobiol* [92]
Cognitive function	↓Working memory performance after hydrocortisone administration	Impaired prefrontal cortex-related executive function	Entringer et al. 2009 *Behav Neurosci* [93]
Cellular aging	↓ Leukocyte telomere length	Risk for age-related degenerative disorders	Entringer et al. 2011 *PNAS* [94]

BMI: body mass index; HDL: low-density lipoprotein; VLDL: very low-density lipoprotein; ACTH: adrenocorticotrophic hormone; PHA: phytohemagglutinin; TH: T-helper cell; IL: interleukin.

these reasons, it is important to gain a better understanding of the origins of individual differences in the propensity for weight and fat mass gain, in order to predict obesity risk and develop strategies for primary prevention [102].

6.1. Targets of Programming of Obesity: Potential Role of the Maternal-Placental-Fetal Endocrine and Immune/Inflammatory Pathway. It is well established that the primary targets of programming of body composition, metabolic function, and obesity risk are the neural networks that regulate energy balance (appetite, feeding, and basal energy expenditure) and peripheral organs and tissues involved in fat synthesis/breakdown, storage and metabolic function (adipocyte, liver, pancreas, and muscle). In this section, we consider and review findings that pertain to the potential role of prenatal stress biology in programming these major targets of interest (see Figure 1).

Stress-related endocrine and immune processes in human pregnancy are associated with not only fetal development and birth outcomes but also with later disease risk. For example, we have reported that placental CRH concentrations in human pregnancy significantly predict the rate of fetal growth and size at birth [104], which, in turn, is a significant predictor of childhood and adult adiposity [105–107]. Other researchers have found a positive association between CRH levels in pregnancy and an increase in central adiposity [108] and alterations in adiponectin levels in 3-year-old children [109]. Yet others have reported a positive association between maternal levels of interleukin-6 (IL-6) in pregnancy and neonatal adiposity [110]. In a recent large epidemiological study in humans, Li et al. [101] found an association between maternal bereavement from death of someone close during pregnancy and an increased risk of overweight in the offspring in later childhood. Furthermore, animal studies have demonstrated long-term effects of prenatal stress exposure on increased body weight in the offspring [24, 111].

6.1.1. Neural Circuits. The central role of ventromedial hypothalamic (VMN) circuits in regulating feeding and energy balance is well established. VMH neurons contain receptors for and receive afferent signals related to fat stores (leptin), nutrient metabolism (insulin), hunger (ghrelin), and satiety (peptide YY), and they integrate peripheral signals of effectors of food intake and energy expenditure so as to prevent substantial variations in the level of energy balance [112]. Also involved in the regulation of appetite and food intake are brain regions that make food intake rewarding (limbic structures), and higher cortical structures (e.g., prefrontal cortex) that are important for learned patterns of eating behavior and executive control [96]. A growing body of the literature suggests that intrauterine perturbations can produce reorganization of these neural pathways that regulate energy intake and expenditure in ways that enhance the development of obesity. Several studies have convincingly demonstrated that biological (endocrine, immune) stress during gestation, triggered by a variety of nutritional, inflammatory, vascular, behavioral, or psychosocial perturbations, can promote obesity in the offspring by reorganizing central neural pathways through programming of energy balance "set points." (see [113] for recent review). One key system involved in the regulation of energy balance is the hypothalamic (CRH)-pituitary (ACTH)-adrenal (cortisol) neuroendocrine stress axis, which forms a network of neuronal pathways capable of interacting with brain circuits controlling energy balance [114]. For instance, the adipogenic hormone leptin which is the afferent loop informing the hypothalamus about the states of fat stores, participates in the expression of hypothalamic CRH, interacts at the adrenal with ACTH, and is regulated by cortisol. Cortisol increases leptin secretion and limits CNS leptin-induced efferents [115].

6.1.2. Adipocytes. Obesity is impacted by increases in fat cell number, size, or both. Fetal adipose tissue development is regulated by the complex interaction of maternal, endocrine,

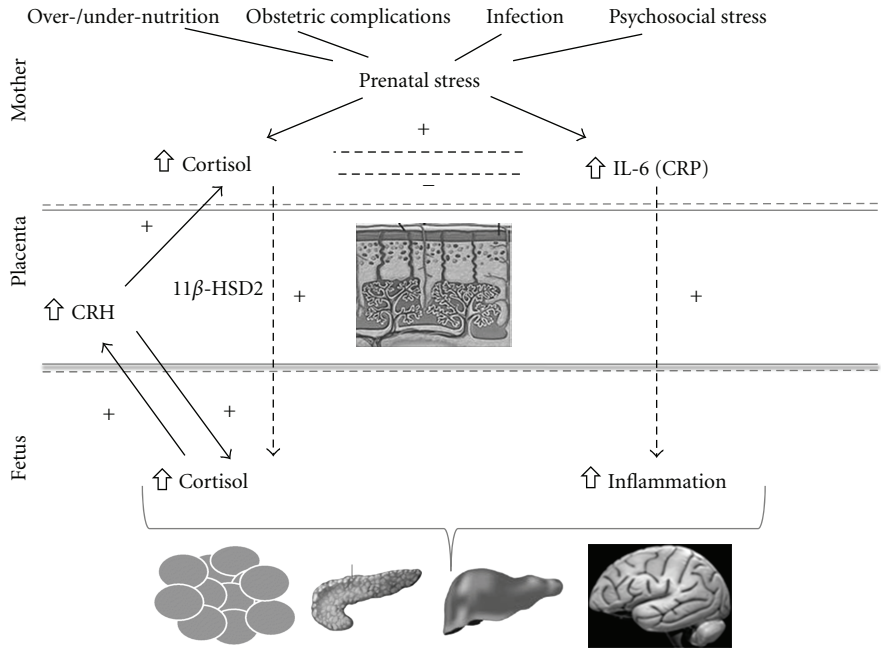

Figure 1: Intrauterine stress biology and programming of fetal targets of body composition and metabolic function. Adverse circumstances during pregnancy (physiological as well as psychological stressors, summarized here as "prenatal stress") have the potential to induce changes in maternal-placental-fetal stress biology (e.g., increases in maternal and fetal cortisol, placental corticotrophin-releasing-hormone (CRH), and inflammatory mediators). The subsequent increase in stress hormones and proinflammatory cytokines in the fetal compartment during sensitive or critical developmental windows can impact the structure and function of the brain and peripheral targets (e.g., adipose tissue, pancreas, and liver) that are related to body composition, energy balance homeostasis, and metabolic function.

and paracrine influences that initiate specific changes in angiogenesis, adipogenesis, and metabolism [116]. Adipogenesis, the process of adipocyte development from mesenchymal stem cell precursors, occurs primarily during late fetal and early postnatal life in humans, and the number of adipocytes is relatively fixed after young adulthood [116–118], supporting the notion that fetal and early postnatal periods are crucial windows in the development of adipose depots. Adipogenesis is highly sensitive to the intrauterine biological environment, in particular to concentrations of insulin-like growth factors, glucose, insulin, and glucocorticoids [116, 117]. *In vitro* studies could show that the differentiation of human adipocyte precursor cells in the presence of insulin is stimulated by cortisol in a dose-dependent manner and occur at physiological concentrations [119, 120]. Furthermore, *in vitro* exposure of isolated human adipocytes to insulin and corticosteroids synergistically induces peroxisome proliferator-activated receptor (PPAR-γ) mRNA expression [121].

CRH seems to be an important regulator of adipocyte function, and CRH receptors are expressed in both white and brown adipocytes [122]. The role of cytokines as regulators of adipose tissue metabolism is well established. Proinflammatory cytokines are elevated in obese individuals, and they seem to modulate leptin secretion from adipocytes [123]. Furthermore, in an animal study prenatal exposure to pro-inflammatory cytokines or dexamethasone had an effect on increased fat depots in the offspring [124]. Animal studies have shown that fat cells exposed to an excess substrate supply during crucial windows in their development have an increased capacity for storing lipid in postnatal life [125, 126]. This enhanced lipogenic capacity renders these individuals more likely to store excess energy in the form of fat and increases their susceptibility to weight gain and obesity and its metabolic sequelae. In individuals exposed to low nutrition levels before birth, adipocyte development is initially sacrificed in favor of "essential" organs [4, 127]. If an *in utero* "restricted" individual is born into a postnatal environment in which nutrient supply is no longer constrained, a period of "catchup" fat deposition ensues, mainly in the visceral adipose depot [128]. These individuals are at increased risk of visceral obesity [107] and, consequently, to the development of insulin resistance and type 2 diabetes [129].

6.1.3. Liver and Pancreas. The liver controls the production and fate of metabolic fuels through the action of hepatic enzymes. Phosphoenolpyruvate carboxykinase (PEPCK), a key enzyme in hepatic gluconeogenesis, is under potent glucocorticoid regulation. In animals, prenatal exposure to dexamethasone produces an increased expression of hepatic glucocorticoid receptors as well as increased levels and activity of PEPCK [130], thereby predisposing these animals to glucose intolerance later in life. Furthermore, manipulation of diet during pregnancy is associated with epigenetic changes in the promotor regions of the genes encoding PPARα and the glucocorticoid receptors in the liver in offspring after birth, thereby altering their metabolic

phenotype [131, 132]. Insulin is produced by the beta cells in the pancreas in response to elevated blood glucose levels. Increased glucocorticoid exposure and malnutrition during fetal development have the potential to permanently reduce the pancreatic beta cell mass and lower pancreatic insulin content, thereby increasing the risk for metabolic disease later in life (reviewed in [133]). For example in humans, prenatal exposure to glucocorticoids or stress was associated with higher insulin resistance in the adult offspring [90, 134].

6.2. Genes, Gene-Environment Interactions, and Epigenetic Mechanisms. The ascertainment of genetic contributors to body composition, obesity, and metabolic dysfunction is an area of active and intense investigation. Although weight and body composition are highly heritable, known genes account for only a modest proportion of their variance [135–137]. Genetic makeup alone cannot explain the rapid increase in obesity prevalence in the population because the genetic characteristics of the human population have not changed in the last three decades, but the prevalence of obesity has tripled during that time [138]. Estimates of maternal transmission of heritability are stronger than those for paternal transmission, which argues in favor of intrauterine effects and/or mitochondrial DNA effects. Moreover, the strongest genetic associations seem to vary as a function of the environment (e.g., effects are seen at specific times but not other times in the life cycle). These observations suggest gene-environment interactions are particularly relevant in the context of the obesity phenotype.

The search for mechanisms by which environmental conditions during development can produce long-term changes in the structure and function of cells, tissues, and organ systems has led to the identification and study of epigenetic processes in the context of fetal programming of health and disease risk. A detailed review of epigenetics is beyond the scope of the current paper, and we have elaborated on this issue elsewhere [139, 140]. The effects of nutritional conditions in early development on epigenetic processes have been studied extensively (for recent reviews see, for example, [6, 141–147]).

We suggest that the incorporation of the genetics and epigenetics of stress and stress biology in the context of fetal programming of body composition, obesity, and metabolic function is likely to yield additional important information that supplements and complements investigations of nutrition-mediated genetic and epigenetic processes underlying fetal programming. As discussed above, many of the effects of maternal nutrition on the developing embryo and fetus may be mediated, in part, by stress hormones such as CRH and cortisol. Diet in prenatal or early postnatal life has been shown to alter the methylation status of several genes implicated in stress and stress physiology, including genes encoding the glucocorticoid receptor and proopiomelanocortin [141]. Conversely, intrauterine or early postnatal exposure to inappropriate levels of stress hormones such as glucocorticoids is known to produce a wide array of epigenetic modifications in tissues including the placenta, brain, adipose tissue, liver, lungs, kidney, skeletal muscle, heart, and blood vessels (for a recent review see [148]). Many of these changes have important long-term implications for body composition and metabolic function. Interestingly, a variant in the gene encoding the glucocorticoid receptor has been associated with increased body fatness in children [149]. We and others have described the association of the same variant with altered physiological stress responses [150]. Furthermore, manipulation of maternal behavior in the early postnatal period in rats permanently alters the offspring's epigenome at the glucocorticoid receptor gene promoter in the hippocampus, and this altered methylation state is associated with changes in GR expression and hypothalamic-pituitary-adrenal (HPA) responses to stress in the offspring [151].

With respect to the issue of the contribution of stress and stress biology it is very likely that genetic and epigenetic variations will be determined to play an important role in moderating the association between intrauterine stress and obesity and metabolic dysfunction risk via maternal-fetal gene-gene and gene-environment interactions at multiple levels, originating with the likelihood of encountering stressful life circumstances, and culminating in modifying the effects of stress-related biological processes on relevant target tissues. For example, women who are carriers of certain genotypes (high-risk alleles in dopamine-related genes) may be more likely to place themselves in stressful life circumstances [152–154]. The psychological appraisal of potentially stressful circumstances may be influenced directly by the maternal genotypic variation (e.g., in the serotonin transporter gene [155]) or indirectly by the fetal genotype (via its effect on alterations in maternal physiology that, in turn, influence maternal psychological appraisals). Next, the ensuing effects of maternal stressful experience on maternal and fetal biology may be moderated by the genetic and epigenetic characteristics of the mother and fetus (e.g., variants in the glucocorticoid receptor gene [156]), respectively. Finally, the effects of stress-related physiological alterations on target placental and fetal tissues implicated in energy balance homeostasis and metabolic function may be further influenced by the genetic and epigenetic makeup of the mother and fetus, respectively.

To date, only a small number of studies have systematically addressed the issue of a genetic predisposition for susceptibility to psychosocial stress and related psychobiological states. For example, we and others have described that certain polymorphisms in the promotor region of the gene encoding the glucocorticoid receptor are associated with changes in the regulation of the HPA axis at different levels including basal level, feedback regulation, and response following a psychosocial stressor (summarized in [156]). Because several genes that code for proteins involved in the regulation of the stress response also are involved in the physiology of pregnancy and fetal development (e.g., CRH, cortisol, IL-6, etc.), individual differences in genetic variation may be another factor underlying susceptibility in terms of the potentially adverse effects of maternal stress on pregnancy outcomes. The participation of placental CRH as a central molecule in regulating various aspects of pregnancy, fetal development, and birth outcomes has been discussed in the preceding sections. Thus, DNA sequence variations in

the CRH gene, the CRH receptor genes, the glucocorticoid receptor gene, and other genes encoding key enzymes and binding proteins in their biosynthetic pathways may have important implications in this context.

Genetic and epigenetic mechanisms have been proposed to explain the observed racial/ethnic disparities in obesity and metabolic diseases, particularly with respect to the hypothesized contribution of prenatal stress. These observed racial/ethnic disparities are commonly assumed to reflect the burden of adverse societal conditions associated with minority racial/ethnic status in the US. Prenatal stress is a plausible mediator of the effects of race/ethnicity via one or both of two possibilities: greater cumulative exposure to stress and greater vulnerability to the effects of stress (arising from differences in psychobiological responses to stress). The characterization of racial/ethnic differences in DNA sequence or epigenetic variation in genes associated with the stress response will prove particularly informative in this regard. For instance, we and others have previously reported significant racial/ethnic differences in stress-related hormonal states in human pregnancy [157, 158]. These racial/ethnic differences in neuroendocrine function in pregnancy may, in turn, reflect one or more of three possibilities: first, that particular genetic variations associated with pathophysiology are more frequent in specific racial/ethnic populations. Second, that there are no differences in the frequency of particular genetic variations across population subgroups; however, they are phenotypically expressed only under certain environmental conditions or exposures associated with particular racial/ethnic populations (e.g., high stress, reproductive tract infection, social or cultural behavioral practices). And third, that there are no differences in the frequency of particular genetic variations across population subgroups; however, specific gene regions are preferentially expressed or silenced by epigenetic modifications that occurred during sensitive or critical periods of the mother's own development under environmental conditions associated with particular racial/ethnic populations (e.g., intrauterine exposure to high stress or infection [159]). One of our ongoing projects is in the process of evaluating these possibilities by determining whether the population structure and functional significance of maternal-fetal genetic variation and gene-environment interactions vary as a function of race/ethnicity.

7. Future Directions: Implications for Research and Clinical Practice

By incorporating the developmental programming approach into the traditional paradigm of causation of complex common health disorders, the focus shifts to placing a far greater emphasis on the health and well being of young women of reproductive age prior to conception and across gestation, in order to more effectively address health and disease risk-related issues in their offspring from infancy and childhood through adolescence and into adult life. A multilevel approach is required that includes molecular and cellular studies, the use of appropriate animal models, and well-designed human studies. In the context of human research, opportunities are limited for experimental manipulations of prenatal stress and the intrauterine environment, and for access to many of the target tissues of interest, particularly in fetal life. Hence, for future research purposes, the value of prospective, longitudinal, follow-up studies, ideally starting before conception, and extending through pregnancy and birth into childhood and beyond, is emphasized. For these studies, deployment of state-of-the art methods, including the assessment of metabolomic and gene expression profiles to precisely characterize maternal nutritional biomarkers and their interactive effects with stress biology during pregnancy, 3D/4D fetal ultrasonography for quantification of fetal growth (biometry), regional blood flow (uterine, umbilical, and cerebral), hepatic and renal volume [160, 161], growth trajectory of organs (placenta, brain, liver, kidneys, and adrenals [162, 163]) and body composition (arm, thigh, and visceral fat/lean mass [164, 165]), coupled with reliable assessments in newborns, infants and children of body composition (with magnetic resonance imaging (MRI) or dual energy X-ray absorptiometry (DXA)) and energy expenditure (basal metabolic rate and total energy expenditure using indirect calorimetry and the doubly labeled water method (DLW), resp.), will move the field forward in an informed manner. Furthermore, recent advances in imaging techniques will likely enable the developments of protocols in infants and children for subcutaneous and visceral fat quantification (especially intrahepatic fat) [166], and characterization of and differentiation between white and brown adipose tissue [167]. These observational studies, in conjunction with parallel molecular studies including studies of human placental, multipotent (stromal) stem cells, and adipose tissue culture systems [168], and coupled with state-of-the-art statistical modeling techniques for parametric and nonparametric repeated measures, time-series data [169–172], will contribute to further defining technical capabilities in this field.

Regarding clinical implications, it is apparent that current approaches to the prevention and management of obesity and associated metabolic disorders have yielded only very limited success. Once an individual becomes obese, it is difficult to lose weight, and even more difficult to sustain weight loss [101–103]; systematic studies of the efficacy of current weight loss programs have provide the sobering statistic that approximately 80–90% of obese people who have lost weight regain it within one year [173–175]. Clearly, it is critical to adopt a developmental framework in order to arrive at a better understanding of the origins of individual differences in the propensity for weight and fat mass gain, and to develop and test hypotheses that set the stage for translational research to inform the subsequent development of primary intervention strategies before an individual becomes overweight or obese, or secondary interventions to increase the likelihood of a favorable and more sustained response to weight loss strategies.

8. Conclusion

Based on the conceptual framework and empirical findings presented here, we suggest that in addition to maternal

nutrition it is important to also consider the potential role of intrauterine stress and stress biology in arriving at a better understanding of developmental programming of health and disease susceptibility. Moreover, we submit that stress-related maternal-placental-fetal endocrine and immune processes in human gestation represent a potentially attractive underlying candidate mechanism for elucidating the common biological basis (pathway) for mediating not only the long-term effects of prenatal stress but also those of a host of other intrauterine perturbations including maternal over- and under-nutrition that have been implicated in this area.

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Research Article

Neonatal SSRI Exposure Programs a Hypermetabolic State in Adult Mice

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Background. Selective serotonin reuptake inhibitor (SSRI) therapy complicates up to 10% of pregnancies. During therapy, SSRIs exert pleiotropic antidepressant, anorexigenic, and neurotrophic effects. Intrauterine SSRI exposure has been modeled by neonatal administration to developmentally immature rodents, and it has paradoxically elicited features of adult depression. We hypothesized neonatal SSRI exposure likewise programs a rebound hypermetabolic state in adult mice. *Methods*. C57BL/6 pups were randomized to saline or sertraline (5 mg/kg/d) from P1–P14. Because estrogen increases tryptophan hydroxylase 2 (TPH2) expression, a subset of female mice underwent sham surgery or bilateral ovariectomy (OVX). Metabolic rate was determined by indirect calorimetry. *Results*. In both male and female mice, neonatal SSRI exposure increased adult caloric intake and metabolic rate. SSRI-exposed female mice had significantly decreased adult weight with a relative increase in brain weight and melatonin excretion, independent of ovarian status. Cerebral cortex TPH2 expression was increased in SSRI-exposed male mice but decreased in OVX SSRI-exposed female mice. *Conclusions*. SSRI exposure during a critical neurodevelopmental window increases adult caloric intake and metabolic rate. Ovarian status modulated central TPH2 expression, but not adult energy balance, suggesting programmed neural connectivity or enhanced melatonin production may play a more important role in the post-SSRI hypermetabolic syndrome.

1. Introduction

The prevalence of depression during pregnancy now exceeds 18%, and more than 13% of pregnancies were complicated by antidepressant therapy in 2003, twice as many as in 1999 [1, 2]. The majority of this increase has come from a heightened utilization of selective serotonin reuptake inhibitors (SSRIs) [2–6]. Among the SSRIs, sertraline remains the most commonly prescribed antidepressant in America [2].

Recent studies have demonstrated detrimental effects of *in utero* SSRI exposure, including fetal growth restriction, neonatal abstinence syndrome, and persistent pulmonary hypertension [5, 7]. The effects of third trimester exposure are often felt to be transient in nature. However, Oberlander and colleagues have demonstrated changes in hypothalamic, cardiovascular, and nociceptive regulation up to 4 months following delivery, and SSRI exposure has now been associated with increased autism risk [8–13].

Murine models of human neurodevelopment are well established, with the first fourteen postnatal days approximating the third trimester of human development [14]. Exposure of male rats to the SSRI citalopram at the later end of this critical window (P8–P21) decreases dorsal raphe tryptophan hydroxylase (TPH) immunoreactivity and elicits behavioral changes consistent with depression [15]. Further studies on that model have identified SSRI-induced disruptions in neural networks and upregulation of the noradrenergic locus ceruleus system [16, 17]. When reported, outcomes have been blunted in female mice and rats [16, 17]; suggesting estrogen-stimulated TPH expression may provide partial protection [18].

We sought to expand the assessment of SSRI-programmed phenotypes and determine the sex-specific effects of neonatal exposure to sertraline, the most commonly prescribed antidepressant. Given the known anorexigenic and sympathoinhibitory effects of acute SSRI administration [19–21], we speculated alterations in energy balance may be a component of a potential "post-SSRI syndrome." We hypothesized that SSRI-exposed male and ovariectomized female mice have decreased adult serotonergic tone that is manifest by increased feed intake and basal metabolic rate.

2. Methods

2.1. Animal Model. Pregnant C57BL/6 mice (Jackson Laboratory, Bar Harbor, ME) were allowed natural delivery and within 12 hours of birth, each litter was adjusted to 6 pups, via culling or adding age-matched pups from other dams. While C57BL/6 dams readily accept cross-fostered pups [22, 23], a recent publication described alterations in adult outcomes when entire litters were cross-fostered at 48 h [23]. A vast majority of litters did not require any additional pups, and the confounding effects of cross-fostering were minimized by randomization. Pups were then randomized within each litter to either receive intraperitoneal saline (10 mL/kg/d) or sertraline (5 mg/kg/d) on days P1–14. This corresponds to a neurodevelopmental window similar to the third trimester in human gestation. The dose utilized was determined from the equation: murine dose = maternal oral dose/maternal body surface area × murine body surface area × oral bioavailability × placental transfer ratio. Utilizing the equation of Meeh for murine body surface area [9.8 × (weight in g)$^{2/3}$] [24], the average weight of mice at 14 d (7 g), the equation of Du Bois for human body surface area [71.84 × (weight in kg)$^{0.425}$× (height in cm)$^{0.725}$] [25], the average weight and height of women in the third trimester (80 kg, 162 cm), 70% oral bioavailability and 29% placental transfer [26], the equation simplifies to murine dose = maternal dose ∗ 0.00039. Thus, to replicate typical low-dose therapy of 100 mg/d, we utilized 0.039 mg/d or ∼5.6 mg/kg/d. To verify the clinical relevance of this dosing regimen, plasma was collected 2 h or 12 h after the final dose of sertraline. Prior to collection, pups were anesthetized with isoflurane (1%), the liver was excised and 600–750 microliter of blood was collected. Plasma was stored at −20°C prior to analysis by gas chromatography at NMS Labs (Willow Grove, PA).

To further assess the protective effect of ovarian function, a subset of 5- to 6-month-old female mice underwent bilateral ovariectomy (OVX) versus sham surgery (ovary visualization without resection) via paravertebral approach under isoflurane anesthesia. Analgesia was provided with flunixin meglumine (2.5 mg/kg once or twice daily), as well as 0.5% bupivacaine along the incisions. A minimum of one month of surgical recovery was provided prior to phenotyping. All surgeries and protocols were in accordance with NIH guidelines and were approved by the Institutional Animal Care and Use Committee at the University of Iowa. All investigations were designed to reduce the numbers of animals required and procedures were modified to lessen or eliminate pain and distress.

2.2. In Vivo Phenotypes. Feed consumption and weight were measured over a 14-day interval while mice received *ad lib* standard rodent chow (4 kcal/g, 6% of energy as fat; 7013; Harlan Teklad, Madison, WI). Basal metabolic rate was then assessed via indirect calorimetry in an airtight thermoneutral chamber. Oxygen consumption (VO2) was calculated while the mice were asleep, as previously described [27]. For male mice, physiologic studies (performed at 5–7 months) were followed by brain harvest under general anesthesia at 6–9 months. The female mice that underwent ovariectomy or sham surgery had delayed euthanasia (11-12 months). During this delay, analysis of the *in vivo* phenotype data led us to place them in a metabolic cage for determination of 24 h urinary melatonin excretion. Samples were stored at −80 degrees until analysis in duplicate for melatonin sulfate (ELISA kit RE54031, IBL Transatlantic). Samples with no detectible melatonin sulfate (lower limit of detection 1 ng/mL) were analyzed with a value of zero. There was one such sample present in each of the 4 groups. Following these studies, female mice were euthanized by organ harvest under general anesthesia, and tissue weights were obtained.

2.3. Tryptophan Hydroxylase Expression. The brain was quickly and bluntly segmented and stored in RNAlater until purification with RNeasy kits (Qiagen, Valencia, CA). Initial coronal sectioning removed the olfactory bulbs anteriorly, as well as the cerebellum and medulla posteriorly. The remaining brain was then sectioned both superiorly and laterally to obtain a sample labeled "cortex" including both the cerebral cortex and pineal gland. The remaining segment, labeled "midbrain" included the dorsal raphe nucleus as well as the diencephalon (thalamus and hypothalamus). Finer dissection was not completed to avoid loss of message due to either passage of time or indiscriminate removal of grossly indistinct regions. RNA was quantitated using a NanoDrop ND-1000 spectrophotometer (Labtech International, East Sussex, UK). Reverse-transcription reactions were performed on 0.5 μg total RNA with the addition of oligo dT, dNTPs, DTT, RNasin, and Superscript III reverse transcriptase (Invitrogen). Quantitative real-time RT-PCR (qPCR) utilized the TaqMan reagent and instrumentation systems (Applied Biosystems, Foster City, CA). Taqman gene expression assay primer/probe sets for mouse Tph1 (assay ID = Mm00493794_m1; context sequence = CCGACCACCCTGGCTTCAAAGACAA), Tph2 (assay ID = Mm00557715_m1; context sequence = TAGACTATTCCAGGAAAAACATGTC), and GAPDH were purchased from Applied Biosystems (Foster City, CA). Since the reaction efficiencies for the 3 assays are matched by design, we used the ΔΔCT method for quantitation.

2.4. Data Analysis. All values other than TPH expression data are presented as mean ± SEM. TPH expression is presented as % control = $100 \times 2^{(-\Delta\Delta CT)}$ with corresponding error bars equal to $100 \times 2^{(-\Delta\Delta CT \pm \text{pooledSE})}$. Statistical comparisons were made using two-way ANOVA and the Holm-Sidak method for multiple pair-wise comparisons. When ANOVA identified a significant interaction between

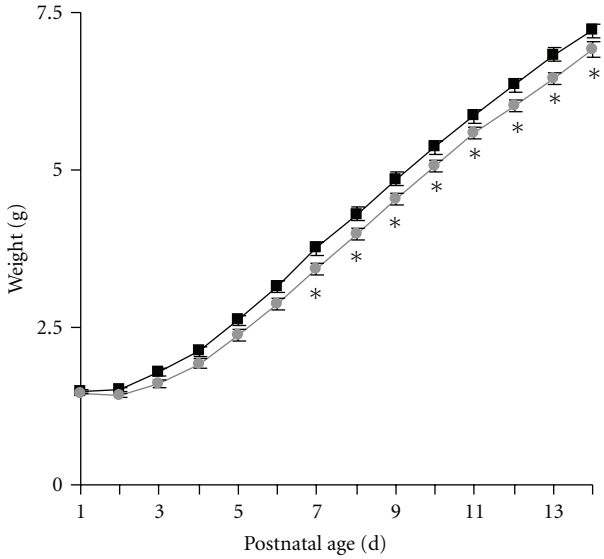

Figure 1: Neonatal SSRI exposure acutely decreased pup growth. Pups were weighed immediately prior to daily administration of 10 mL/kg saline (white symbols) or 5 mg/kg sertraline (gray symbols) from P1 to P14. $N = 34$ control and 40 SSRI-exposed pups. $^*P < 0.05$ by two way repeated measures ANOVA with Holm-Sidak method for multiple pair-wise comparisons.

SSRI exposure and ovarian status, pairwise comparisons were made by the Holm-Sidak method. Postmortem tissue weights were compared by Student's t-test. A value of $P < 0.05$ was considered significant. All analyses were performed using SigmaPlot 12 (Systat Software Inc.).

3. Results

3.1. Acute Sertraline Administration Decreases the Growth of Neonatal Mice and Models Intrauterine Human Exposure. Pup weights at the initiation of sertraline exposure (P1) were similar (control 1.49 ± 0.02 g, SSRI-exposed 1.46 ± 0.02 g). Throughout the final 9 days of injections (P6–P14), sertraline-exposed mice had mild but statistically significant growth restriction (Figure 1, overall ANOVA $P = 0.032$, difference of means 0.26 g). On the final day of exposure (14 d), plasma levels 2 h after injection were 71.8 ± 1.3 ng/mL ($N = 6$) and 12 h after injection were 13.1 ± 0.6 ng/mL ($N = 7$). Based on these levels, there was a half-life 4.1 h, estimated peak concentration 101 ng/mL, and estimated trough concentration of 1.7 ng/mL. Our projected peak concentration approximates that seen in pregnancy (99 ng/mL based on 150 mg/d dosing) [28], and our projected trough approximates umbilical cord levels (4.9 ng/mL) [26]. The mean plasma level in mice (24.8 ng/mL) closely approximates mean human levels measured in the general population (20.4 ng/mL) and in pregnant women (also 20.4 ng/mL) [26, 29].

3.2. Neonatal SSRI Exposure Induces a Hypermetabolic State. Body weight had normalized at the beginning of adult phenotype assessment at 5–7 months (Figure 2(a)). At this point, mice were separated into individual cages to quantify feed intake. Sertraline exposure increased adult male and female caloric intake (Figure 2(b), $P = 0.004$). This increased caloric intake was matched by an increased basal metabolic rate (resting oxygen consumption) in both male and female SSRI-exposed mice (Figure 3, $P = 0.033$). Upon necropsy, SSRI-exposed female and male mice had decreased body weight (Table 1, $P = 0.021$ and $P = 0.048$, resp.) with increased relative brain weight seen in females ($P = 0.017$) but not males ($P = 0.56$). Additional organ weights were not obtained from male mice. Among female mice, there were no significant alterations in the absolute or relative weights of the liver ($P = 0.07$), intraabdominal white adipose tissue ($P = 0.09$), or interscapular brown adipose tissue ($P = 0.85$), although body composition tended to be leaner in the growth-restricted SSRI-exposed mice (Table 1).

3.3. Neonatal Sertraline Exposure Increases Adult Tryptophan Hydroxylase Expression and Melatonin Excretion. In order to assess central serotonergic tone, regional and isoform-specific TPH expression was determined. By qPCR (Figure 4(a)), SSRI-exposed male mice had increased expression of the major TPH isoform (TPH2) within the cerebral cortex (2.8-fold increase, $P = 0.048$) and midbrain (2.5-fold increase, $P = 0.11$). Among female mice, there was an interaction between SSRI exposure and OVX ($P = 0.017$), with significantly decreased TPH2 expression in OVX SSRI-exposed mice cortex ($P = 0.027$). Brain TPH1 mRNA expression was not altered by SSRI exposure or ovarian status (Figure 4(b)). Overall, TPH2 mRNA expression was enriched 143-fold in the midbrain (ΔCT: 4.2 ± 0.3) versus the cortex (ΔCT: 11.3 ± 0.2), while TPH1 mRNA expression was increased 2-fold in the cortex (ΔCT: 10.8 ± 0.1) over the midbrain (ΔCT: 11.9 ± 0.1). Because a vast majority of excreted serotonin metabolites originate from systemic rather central sources, we chose to measure urinary excretion of the stable melatonin metabolite, melatonin sulfate [30]. Unlike serotonin produced within the dorsal raphe, melatonin produced within the pineal gland readily enters the systemic circulation such that urinary excretion allows for noninvasive approximation of central production. Independent of ovarian status, neonatal SSRI exposure increased urinary melatonin sulfate excretion (Figure 5, $P = 0.034$).

4. Discussion

Over the past decade, a growing percentage of infants have been exposed to maternal antidepressant therapy without long-term outcome data. Animal models have been developed to fill this void. Advantages of murine models of human exposures include a shortened lifespan allowing life-course assessment and the availability of isogenic inbred strains. To capitalize on these advantages, a well-timed clinically relevant environmental exposure must be introduced during a critical window of developmental susceptibility. Intrauterine SSRI exposure induces cardiac malformations in mice that

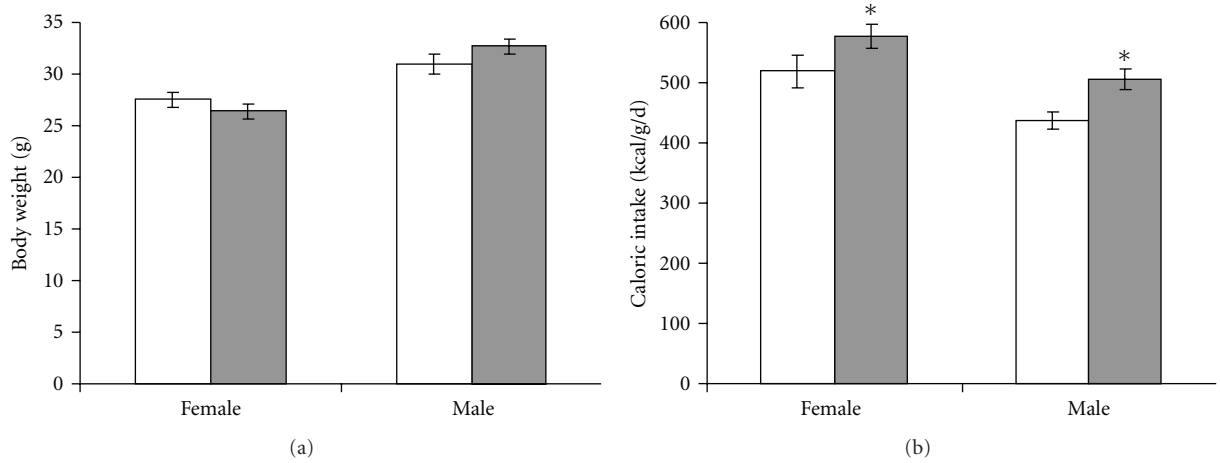

Figure 2: Neonatal SSRI exposure increases adult caloric intake. Intake of standard rodent chow was recorded over a 2 week interval and compared between control mice that received 10 mL/kg/d saline (white bars) and SSRI-exposed mice that received 5 mg/kg/d sertraline (gray bars) from P1 to P14. $N = 27$ control female (16 litters), 24 SSRI-exposed female (14 litters), 14 control male (9 litters), 12 SSRI-exposed male (10 litters); $^*P = 0.004$ versus control by ANOVA.

Table 1: From postnatal day 1 to 14, mice received injections of saline (control) or the SSRI sertraline. After obtaining adult phenotypes, tissue weights were obtained from 11- to 12-month-old female and 6- to 9-month-old male mice. $^*P < 0.05$ versus control by Student's t-test

		Female	Female	Male	Male
		Control	SSRI	Control	SSRI
N		15	14	8	10
Body Weight	(g)	35.3 ± 1.6	29.6 ± 1.7 *	39.7 ± 2.1	35.0 ± 1.0*
Brain	(mg)	457 ± 4	458 ± 4	421 ± 12	406 ± 8
	(mg/g)	13.3 ± 0.6	16.1 ± 0.9 *	10.9 ± 0.7	11.3 ± 0.4
Liver	(mg)	1251 ± 103	1241 ± 70		
	(mg/g)	37 ± 3	42 ± 2		
White adipose	(mg)	2688 ± 308	1761 ± 396		
	(mg/g)	73 ± 6	53 ± 9		
Brown adipose	(mg)	127 ± 12	109 ± 12		
	(mg/g)	3.6 ± 0.3	3.6 ± 0.3		

are reminiscent of the congenital cardiac defects seen with intrauterine SSRI exposure in humans [31–34]. However, the third trimester of human neurodevelopment is best modeled in neonatal mice and rats. In rats, neonatal SSRI exposure decreases synaptogenesis and elicits features consistent with depression [15–17]. This post-SSRI syndrome appeared to have a sexually dimorphic presentation with male mice affected more than females. Our well-powered investigations clarify and extend these studies in demonstrating an SSRI-programmed hypermetabolic state in both male and female mice. Our data further demonstrate that while ovarian function modulates serotonergic tone, females are not sheltered from the effects of neonatal SSRI exposure.

Our dosing regimen (5 mg/kg once daily) was designed to replicate intrauterine exposure to maternal doses of only 100 mg once daily, and it matches the lowest exposure used in analogous preclinical studies [35]. Based on studies showing dose-dependent growth inhibition by sertraline [35], it is possible higher exposures may elicit greater programming effects. Consistent with the results seen in rats, neonatal mice have faster sertraline elimination (half-life 4 h) than young women (half-life 32 h) [36, 37]. We utilized a typical once daily dosing regimen that led to slightly exaggerated peak and trough fluctuations. Further studies with a more frequent dosing interval are needed to determine the programming effects of the absolute exposure versus the pattern of exposure and withdrawal. Notably, intrauterine SSRI exposure-associated neonatal abstinence syndrome appears to increase the risk for persistent social-behavioral abnormalities [5, 38].

The increased feed intake we identified in SSRI exposed adult mice contrasts with the anorexigenic effect of acute SSRI therapy [39, 40]. This is consistent with the developmental origins "predictive adaptive responses" theory or the broader developmental biology principle of phenotypic plasticity [41]. Both fields agree that when normal physiologic processes are perturbed by environmental exposures, the organism adapts to restore homeostatic balance. However, if the exposure ceases after this window of developmental

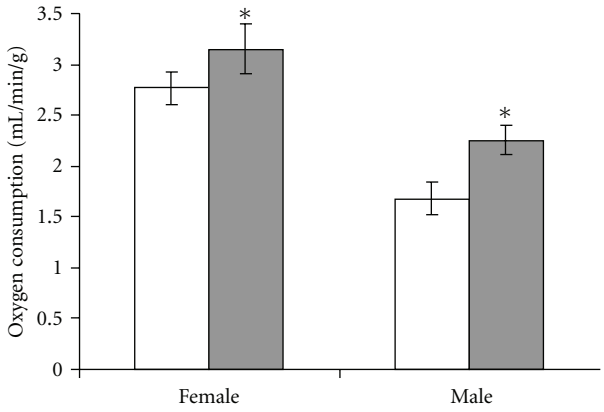

Figure 3: Neonatal SSRI exposure increases adult basal metabolic rates. Control (white bars) and SSRI-exposed mice (gray bars) were placed in a thermoneutral environment and oxygen consumption rates were measured during sleep (nadir in oxygen consumption). $N = 27$ control female (17 litters), 24 SSRI-exposed female (15 litters), 12 control male (9 litters), 13 SSRI-exposed male (10 litters); $^*P = 0.033$ versus control by ANOVA.

plasticity closes, the adaptive response may be fixed and exert maladaptive effects. The remainder of our investigations sought to unravel the developmental origins of this SSRI-induced adult hyperphagia.

Intriguingly, despite the increased caloric intake of SSRI-exposed mice, body weight was either maintained or decreased compared to saline-exposed controls. While the concomitant increase in metabolic rate seen in SSRI-exposed mice may be explanatory, further studies are necessary to define whether the increased feed intake is a primary alteration or a reaction to the increased energy utilization. The diminished weight gain seen in SSRI-exposed mice between baseline assessment and necropsy is consistent with their hypermetabolic state. A cause-effect relationship would be strengthened through investigation of alternative explanations, including the potential that neonatal SSRI exposure increases the stress/catabolic response to phenotypic assessment or the stress/hyperactivity response to isolation within the home cage. The SSRI-induced hypermetabolic state (increased resting expenditure) is itself a novel finding with a number of potential etiologies. Notably, the lean body habitus induced by neonatal SSRI exposure may both account for the increase in metabolic rate (as indexed by body weight) and be a manifestation of the hypermetabolic state. Among factors known to increase metabolic rate, activation of the adrenosympathetic or thyroid axes merit further consideration, especially in light of observational studies that have found an association between intrauterine SSRI exposure and postnatal hypothalamic alterations [13].

To further explore the molecular etiology for the persistent hypermetabolic state, we assessed cortical and midbrain TPH expression. SSRI-exposed male mice had a dramatic increase in expression of TPH2. While statistical analysis only reached significance in the cortex, similar effects were apparent in the midbrain. Consistent with studies in humans [42], we detected lower TPH2 expression in the cerebral cortex then in the midbrain section containing the dorsal raphe nucleus. Although there is currently no evidence this cortical expression substantially contributes to central serotonin synthesis, increased cortical TPH expression and activity has been associated with suicide in humans and altered stress hormone levels in rats [43, 44]. Further studies with region-specific TPH2 overexpression or inactivation will be needed to assess the effects of cortical TPH2 on central serotonin levels and behavioral phenotypes. Based on studies showing dose-dependent inhibition of TPH by SSRIs, it is also possible higher or more frequent exposures would also have greater effects on adult TPH expression and metabolic parameters.

Our results in male mice are in stark contrast with the reduced TPH immunoreactivity seen in the rat dorsal raphe nucleus following citalopram administration from P8–P21. Potential explanations for the discrepancy include: differences in mRNA versus protein expression (e.g., presence of RNA interference or instability), SSRI-specific or species-specific effects, and vastly different windows of exposure. Our data showing increased TPH2 after sertraline exposure is consistent with the increase in TPH mRNA and protein expression seen during sertraline or fluoxetine administration to rats [45]. Although parallel changes in mRNA and protein have been reported following sertraline exposure, it is possible that TPH1 and TPH2 are regulated at both transcriptional and translational levels. Recently developed TPH1 and TPH2 antibodies may permit analysis of isoform-specific expression with future cohorts of mice [46]. Interestingly, during acute citalopram administration, there is a decrease in TPH2 mRNA that does not translate to a change in protein levels [47], highlighting the importance of SSRI-specific research.

In our investigations, there was a clear impact of sex and a lesser effect of ovarian status on SSRI-induced TPH2 expression, suggesting the programmed alterations in TPH2 expression were likely established before pubertal development. Notably, we previously demonstrated male mice have a delayed neonatal window of developmental susceptibility [27], consistent with the female advantage seen following premature delivery [48]. It is possible an earlier window of exposure would be necessary to induce similar programming effects in females.

Our study is one of the first investigations to report long-term outcomes in SSRI-exposed female as well as male mice. Other than TPH2 mRNA expression and adult brain weights, very similar phenotypes were seen in male and female mice, in contrast to the sex-specific differences often reported in undernutrition or reduced uterine perfusion models of metabolic and cardiovascular programming [27, 49, 50]. Because the gene expression studies were not completed at the same age in male and female mice and the male mice did not undergo survival surgery, direct comparison of male and female TPH expression patterns are difficult. Regardless, the lack of correlation between central TPH2 expression and the hypermetabolic aspect of the post-SSRI syndrome led us to explore alternative etiologies.

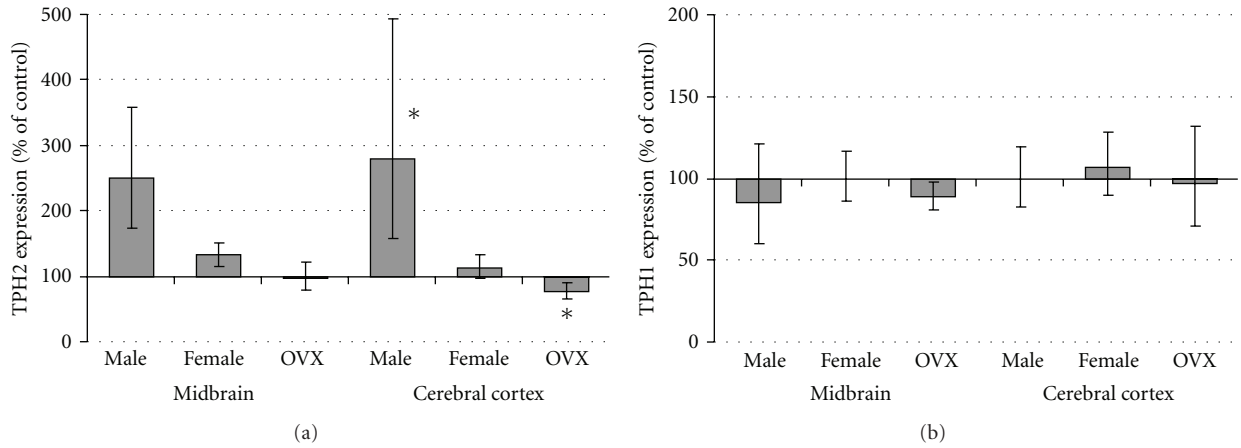

Figure 4: Cerebral cortex expression of TPH2 mRNA is increased in SSRI-exposed male mice. PCR was performed on midbrain and cerebral cortex homogenates obtained from control and SSRI-exposed mice (gray bars). Expression of the two tryptophan hydroxylase isoforms (TPH2 and TPH1) was normalized by GAPDH and ΔΔCT values were calculated to determine relative mRNA abundance. $N = 5$–10 mice from 5–8 litters per group; $^*P < 0.05$ versus control by ANOVA with the Holm-Sidak method for multiple comparisons.

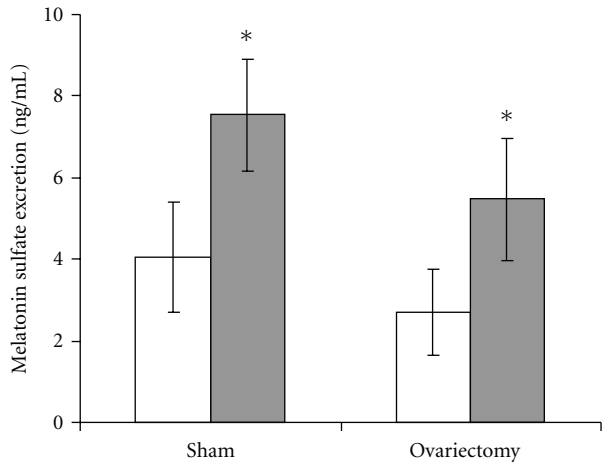

Figure 5: Urinary excretion of the major melatonin metabolite is increased by neonatal SSRI exposure independent of ovarian status. Mice were placed in metabolic cages for 24 hours and urinary excretion of melatonin sulfate was quantified by ELISA for samples from control (white bars) and SSRI-exposed mice (gray bars) that had undergone sham surgery or bilateral ovariectomy at least one month prior to collection. $N = 5$–9 mice from 5–7 litters per group; $^*P = 0.034$ versus control by ANOVA, $P = 0.23$ for ovariectomy versus sham surgery.

Prior studies suggested TPH1 is expressed in the cortex during neurodevelopment and we hypothesized SSRI exposure might trigger a persistence of this immature expression pattern [51]. While ours confirmed an increased expression in the cortex and pineal sample relative to that in the midbrain, there was no significant programming effect seen. Further studies are needed focused on pineal gland mRNA and protein expression of enzymes within the melatonin synthetic pathway.

We were interested in melatonin for a number of reasons. Melatonin and serotonin share metabolic pathways including tryptophan hydroxylase conversion of tryptophan to the common precursor, 5-hydroxytryptophan. Exogenous melatonin has demonstrated efficacy in animal models of obesity with weight loss attributed to an increase in metabolic rate, potentially through noradrenaline sensitization or increased mitochondrial respiration [52–54]. The increased melatonin sulfate excretion seen in female mice following neonatal SSRI exposure may thus contribute to the associated hypermetabolic state. Because caloric intake, metabolic rate and TPH1 expression did not show sex-specific differences, additional male mice were not generated for the determination of urinary melatonin excretion. Notably, TPH is not the rate-limiting enzyme in melatonin production. Further studies, including microdialysis, are needed to define the circadian pattern of serotonin and melatonin production within discrete brain regions. Likewise, serotonin receptor antagonists and/or pinealectomy would help disentangle the importance of these signaling pathways in programmed mice.

In conclusion, the exposure of neonatal mice to the most commonly prescribed SSRI led to a post-SSRI syndrome associated with a hypermetabolic state, an upregulation of TPH expression in male mice, and increased melatonin excretion in female mice. The anorexigenic effects of serotonin and melatonin, as well as the lack of obesity in male and female mice, suggest the hyperphagia is not a primary phenotype, and it may be a compensation for the increased metabolic rate. In addition to translational mechanistic studies, clinical assessments are critically needed to assess the metabolic and neuroendocrine status of infants inadvertently exposed to maternal SSRI therapy.

Authors' Contribution

G. J. Kummet and S. E. Haskell contributed equally to this work.

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Research Article

Maternal Hyperglycemia Disrupts Histone 3 Lysine 36 Trimethylation of the IGF-1 Gene

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In utero environmental adaptation may predispose to lifelong morbidity. Organisms fine-tune gene expression to achieve environmental adaptation by epigenetic alterations of histone markers of gene accessibility. One example of epigenetics is how uteroplacental insufficiency-induced intrauterine growth restriction (IUGR), which predisposes to adult onset insulin resistance, decreases postnatal IGF-1 mRNA variants and the gene elongation mark histone 3 trimethylation of lysine 36 of the IGF-1 gene (H3Me3K36). Limitations in the study of epigenetics exist due to lack of a primary transgenic epigenetic model. Therefore we examined the epigenetic profile of insulin-like growth factor 1 (IGF-1) in a well-characterized rat model of maternal hyperglycemia to determine if the epigenetic profile of IGF-1 is conserved in disparate models of *in utero* adaptation. We hypothesized that maternal hyperglycemia would increase IGF-1 mRNA variants and H3Me3K36. However maternal hyperglycemia decreased hepatic IGF-1 mRNA variants and H3Me3K36. This finding is intriguing given that despite different prenatal insults and growth, both maternal hyperglycemia and IUGR predispose to adult onset insulin resistance. We speculate that H3Me3K36 of the IGF-1 gene is sensitive to the glucose level of the prenatal environment, with resultant alteration of IGF-1 mRNA expression and ultimately vulnerability to adult onset insulin resistance.

1. Introduction

An adverse *in utero* nutritional environment predisposes to lifelong morbidity [1]. One way an organism can adapt to its adverse *in utero* nutritional environment is through fine-tuning gene expression. The modification through which reprogramming of gene expression occurs is called epigenetics. The epigenetic profile consists of a series of DNA and histone modifications that allow increased or decreased access of transcription machinery to DNA for fine-tuning of gene expression. Fine-tuning of gene expression and environmental adaptation can occur through alternate promoter or exon usage.

IGF-1 exemplifies a classic gene that undergoes epigenetic regulation [2, 3]. IGF-1 is a gene whose protein is responsible for postnatal growth and is involved in insulin sensitivity and can be transcribed from one of two promoters and with alternative splicing of exon 5 (Figure 1). Regulation of IGF-1 variant expression is in part due to alteration of the pattern of DNA methylation and histone modifications around the gene and is sensitive to the perinatal environment [3–5].

Hepatic IGF-1 mRNA levels largely impact serum IGF-1 levels and are heavily modulated by epigenetics in the setting of intrauterine growth restriction (IUGR) [3, 6]. IUGR causes fetal hypoglycemia, decreases postnatal growth, and predisposes to adult onset insulin resistance, both of which are modulated by IGF-1. Observation of the epigenetic profile of the IGF-1 gene from promoter 1 through the 3′ untranslated region (UTR) at day of life (DOL) 21, prior to the onset of insulin resistance, revealed persistent decrease in histone 3 trimethylation of lysine 36 (H3Me3K36) [3], a histone mark that is associated with gene elongation [7–9]. Although the effects of IUGR on the epigenetic profile of the IGF-1 gene and H3Me3K36 in particular are known, further study of the epigenetic profile of the IGF-1 gene has

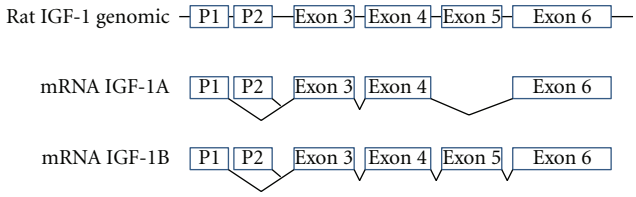

Figure 1: Genomic and mRNA variants of the rat IGF-1 gene. Exons are shown as boxes and introns as lines. IGF-1A lacks exon 5, while IGF-1B contains exon 5. Transcription of IGF-1A and IGF-1B may begin from within either of promoter 1 (P1) or promoter 2 (P2).

been limited due to lack of a primary transgenic epigenetic model. Therefore we sought an alternative method to study the epigenetic profile of IGF-1, using a disparate model to IUGR to determine whether the vulnerability of H3Me3K36 to perinatal insults is conserved.

The model we chose to study epigenetic profile of the IGF-1 gene is a well-characterized model of streptozotocin- (STZ-) induced maternal hyperglycemia [10]. Maternal hyperglycemia was chosen because the *in utero* effects of maternal hyperglycemia on the fetus are quite different from those seen in the setting of IUGR. In humans, maternal hyperglycemia often generates large for gestational age infants and increases IGF-1 serum levels at birth [11–13], yet it still predisposes to alterations in postnatal growth and to adult onset insulin resistance. In this rat model of maternal hyperglycemia, offspring of hyperglycemic mothers (OHMs) have the same average birth weight but with greater numbers of larger and smaller pups than offspring from control (CON) mothers, OHM males gain less weight after 2 months of age, and OHM males develop insulin resistance by 6 months of age [10]. DOL 21 was chosen for our epigenetic profile evaluation of the IGF-1 gene because it precedes the confounders of adolescence, weight gain, and insulin resistance.

We hypothesized that maternal hyperglycemia in rats would increase offspring serum IGF-1 levels, hepatic IGF-1 mRNA variants, and epigenetic markers of IGF-1 associated with gene elongation. Specifically, rat maternal hyperglycemia would decrease offspring promoter DNA methylation and alter multiple markers of the histone code from promoter 1 through the 3′UTR of the IGF-1 gene including increased H3Me3K36. Further, because of their increased risk of development of insulin resistance in adulthood, we hypothesized that these alterations of IGF-1 will be worse in males.

2. Materials and Methods

2.1. Animals. Frozen rat serum and liver were generously given by Dr. Segar at the University of Iowa. Animals from different litters were used for each experiment with an $n = 3$–6 per sex and per treatment for a total of 24 animals for serum glucose and IGF-1 experiments and a total of 15 animals for liver mRNA and epigenetic profile experiments. Animal procedures were previously described [10]. In brief, there was no difference in litter size between OHM and CON [10]. Offspring birth weights did not differ between OHM and CON, though with greater variability among OHM such that there were more large and small pups. Single serum glucose was determined at necropsy using the LifeScan One Touch Ultra Blood Glucose Monitoring System (LifeScan Inc, Milpitas, CA), for a total of $n = 6$ per sex per treatment.

2.2. Enzyme Immunoassay. Serum from DOL 21 OHM was generously given by Dr. Segar at the University of Iowa. Serum IGF-1 levels at DOL 21 were measured in triplicate with the Quantikine Mouse/Rat IGF-1 enzyme immunoassay kit following the manufacturer's protocol (R&D Systems, Inc., Minneapolis, MN, USA).

2.3. RNA Isolation. Total RNA was isolated from frozen liver at DOL 21 as previously described [14] using the Nucleospin RNAII kit (Machery-Nagel, Bethlehem, PA, USA), including DNase I treatment. RNA was quantitated with a spectrophotometer and checked by gel electrophoresis for integrity. The cDNA was synthesized from 1 μg RNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) per manufacturers' protocol.

2.4. Real-Time Reverse Transcriptase Polymerase Chain Reaction (RT-PCR). Real-time RT-PCR was performed as described previously [14]. Primers and probes were the same as used previously [3]. Semiquantitative real-time RT PCR quantification was then performed using glyceraldehydes-3 phosphate dehydrogenase (GAPDH) as an internal control, as Ct values of GAPDH did not differ between CON and OHM animals [15]. Relative quantification of PCR products was based on differences between GAPDH and the target using the comparative Ct method (TaqMan Gold RT-PCR manual; PE Biosystems, Foster City, CA, USA).

2.5. Chromatin Immunoprecipitation (ChIP) Assay and Real-Time PCR. ChIP with 1 μL anti-H3AcK14, anti-H3Me2K4, anti-H3Me3K4 (Millipore Upstate, Charlottesville, VA, USA), and anti-H3Me3K36 (Abcam, Cambridge, MA, USA) was performed as described previously for chromatin from DOL 21 livers [3, 14]. Chromatin equivalent to 100 μg DNA based on A260 absorption was used in each immunoprecipitation (IP) reaction. After purification from IP chromatin, DNA was determined with a standard curve with SYBR Safe DNA gel stain (Molecular Probes, Eugene, OR, USA). The SYBR Safe fluorescence was measured using a Tecan plate reader (Genios Pro-Basic w/o FP; Tecan Austria GmbH, Grodig, Austria) and Magellan V 6.2 software (Tecan). DNA fragments containing IGF-1 site-specific sequences including the seven regions of interest (P1, P2, exon 3, exon 4, exon 5, and proximal and distal 3′untranslated region (UTR) of exon 6) of the IGF- 1 gene were quantified by real-time PCR [3]. Primer and probe sequences are the same as described previously [3], and for exon 3 the following primers were used: forward primer AGACGGGCATTGTGGATGA, reverse primer TCCTGGGTGTGCCTTTGAC, and probe

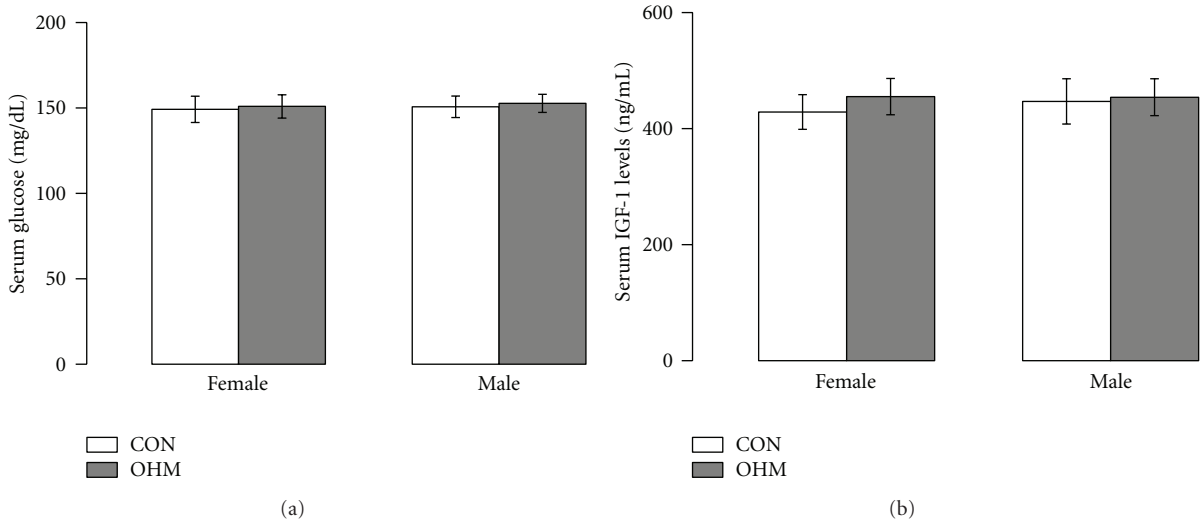

Figure 2: Maternal hyperglycemia did not decrease offspring glucose and serum IGF-1 levels. (a) DOL 21 OHM glucose levels for female and male offspring, CON in white bars and OHM in grey bars. (b) DOL 21 OHM serum IGF-1 levels for female and male offspring, CON in white bars and OHM in grey bars. Serum glucose and IGF-1 levels were expressed as mean ± standard error of the mean, $n = 6$ CON female, $n = 6$ OHM female, $n = 6$ CON male, and $n = 6$ OHM male.

TGTTGCTTCCGGAGCT. Relative quantification of PCR products was based on value differences between the target and intergenic control using the comparative Ct method [15]. An intergenic region upstream of the IGF-1 gene was chosen as a well-characterized nontranscribing region of DNA that contains the histone marks of interest in this series of experiments as described by Fu et al. [3]. The distribution pattern of histone modifications along IGF-1 was determined by looking at the seven sites as indicated previously and expressed as a percentage of the values obtained for P1.

2.6. Bisulfite Modification. Bisulfite modification was performed as described previously using the primers as described previously [3, 14]. PCR conditions were 95°C for 10 minutes, then 94°C for 30 seconds, annealing at either 53°C or 54°C for 30 seconds depending on the primers, and 72°C for 30 seconds, for 35 cycles. PCR products were cloned into the vector for pSC-A (Stratagene, Cedar Creek, TX, USA). Six to eight colonies from each PCR cloning reaction were inoculated into SeqPrep 96 well plates (Edge BioSystems, Gaithersburg, MD, USA). The plasmid DNA was prepared using the SeqPrep 96 Plasmid Prep Kid (Edge BioSystems) and sequenced according to protocol using the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems) with M13 forward or reverse primers.

2.7. Statistics. Data were presented as a mean ± standard error (SE) percent of control or of P1. ANOVA (Fisher's protected least-significant difference) and Student's unpaired t-test were used for real-time RT PCR. Student's 2-tailed t-test was used for DNA methylation. A value of $P < 0.05$ was considered to be statistically significant.

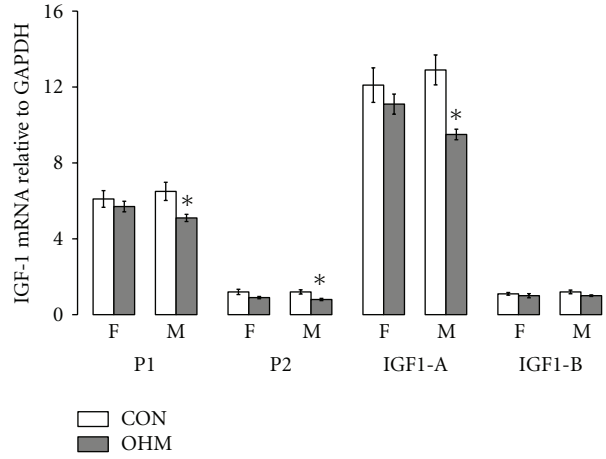

Figure 3: Maternal hyperglycemia decreased hepatic IGF-1 mRNA variants in males. DOL 21 OHM hepatic mRNA variants were expressed as mean ± standard error of the mean relative to GAPDH, for female (F) and male (M) offspring, CON in white bars and OHM in grey bars, $n = 4$ CON female, $n = 3$ OHM female, $n = 5$ CON male, and $n = 3$ OHM male. $^*P < 0.05$.

3. Results

3.1. Hepatic IGF-1 mRNA and Serum Levels. No difference in serum glucose and IGF-1 levels was detected between OHM and CON at DOL 21 (Figure 2). Maternal hyperglycemia significantly decreased male promoter 1 (P1), promoter 2 (P2), and IGF-1A mRNA levels (Figure 3). No changes were seen in female IGF-1 mRNA levels (Figure 3).

3.2. Hepatic IGF-1 Histone Code. Seven sites along the hepatic IGF-1 gene were analyzed for four histone H3 covalent modifications in the control group to determine a normal histone code along the hepatic rat IGF-1 gene. These same

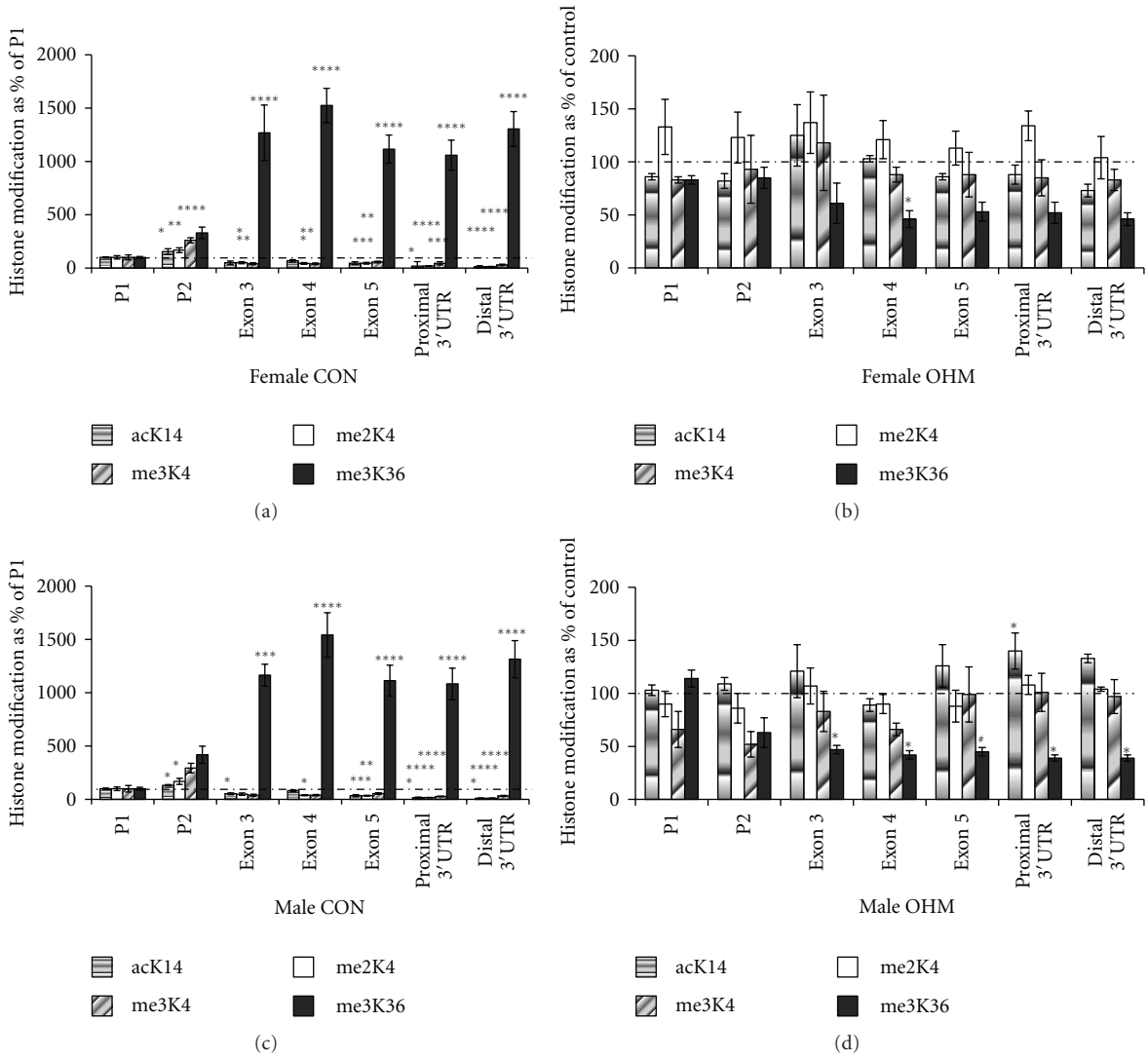

Figure 4: Maternal hyperglycemia decreased H3Me3K36 along the length of the IGF-1 gene in males. (a) Female IGF-1 histone modifications in control animals expressed as a mean percent of P1 ± standard error of the mean. (b) Female IGF-1 histone modifications in OHM animals expressed as a mean percent of gender matched control values ± standard error of the mean. (c) Male IGF-1 histone modifications in control animals expressed as a mean percent of P1 ± standard error of the mean. (d) Male IGF-1 histone modifications in OHM animals expressed as a mean percent of gender matched control values ± standard error of the mean, $n = 4$ CON female, $n = 3$ OHM female, $n = 5$ CON male, and $n = 3$ OHM male. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$.

seven sites were analyzed in OHM to determine the effect of maternal hyperglycemia on these sites. Each modification in both CON and OHM groups was normalized to the intergenic region. Each modification was then expressed as a percent of P1.

At DOL 21, acetylation at lysine 14 is increased at P2 and then decreased along the rest of the IGF-1 gene relative to P1 in both genders. Similarly, di- and trimethylation are increased at P2 and decreased along the remainder of the IGF-1 gene in both genders. Trimethylation of lysine 36 was increased at all sites relative to P1 in both genders (Figure 4).

These seven sites were also analyzed in the offspring of hyperglycemic dams. Results were presented relative to controls where controls were considered to be 100%. At DOL 21, maternal hyperglycemia increased acetylation of lysine 14 in males distally, with no change seen in females. No change was seen in either di- or trimethylation of lysine 4 in either gender. Maternal hyperglycemia decreased trimethylation of lysine 36 throughout the IGF-1 gene in males, and at exon 4 in females (Figure 4).

3.3. IGF-1 P1 Methylation. Twelve CpG sites within P1 of IGF-1 were analyzed for methylation. Maternal hyperglycemia significantly increased methylation in females at sites −260 and −143. No difference was seen in methylation at P1 in males (Figure 5).

3.4. IGF-1 P2 Methylation. Six CpG sites within P2 of IGF-1 were analyzed for methylation. No significant differences were seen in either gender (Figure 5).

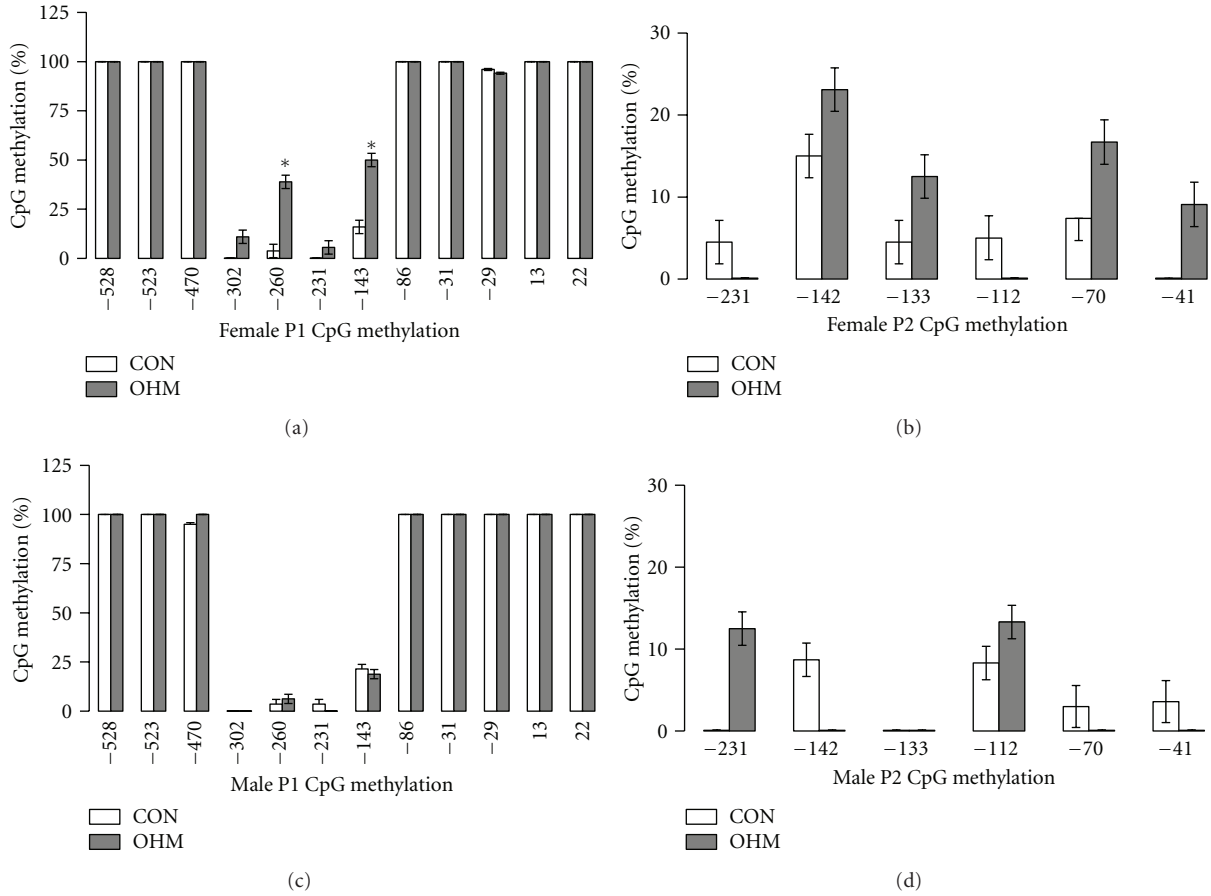

Figure 5: Maternal hyperglycemia increased rat promoter 1 CpG methylation in females. (a) Rat promoter 1 CpG methylation in females. (b) Rat promoter 2 CpG methylation in females. (c) Rat promoter 1 CpG methylation in males. (d) Rat promoter 2 CpG methylation in males. All data were expressed as mean percent of methylation ± standard error of the mean, $n = 4$ CON female, $n = 3$ OHM female, $n = 5$ CON male, and $n = 3$ OHM male. $^*P < 0.05$.

4. Discussion

The most important finding of this study is that maternal hyperglycemia decreased H3Me3K36 of the IGF-1 gene in the same fashion that IUGR decreased this histone mark [3]. This finding is most intriguing given that both maternal hyperglycemia and IUGR have the same phenotype of adult onset insulin resistance. We speculate that H3Me3K36 of the IGF-1 gene is sensitive to the glucose level of the prenatal environment, with resultant alteration of IGF-1 mRNA expression and ultimately vulnerability to adult onset insulin resistance.

Maternal hyperglycemia decreased hepatic H3Me3K36 of the IGF-1 gene in DOL 21 OHM males. This histone mark is often associated with mRNA elongation and actively transcribed genes [7–9]. The protein that places the H3Me3K36 mark, one example of which is Wolf-Hirschhorn Syndrome Candidate 1, associates itself with RNA polymerase 2, the enzyme responsible for mRNA transcription, and thus influences mRNA levels [9, 16]. H3Me3K36 appears to be particularly sensitive to alteration by glucose given the similarity of findings between our study of OHM and of IUGR [3]. Decreased H3Me3K36 of the IGF-1 gene occurred in both genders in our prior study of IUGR, and these findings were associated with decreased IGF-1 mRNA variant levels and adult onset insulin resistance in both genders [3, 17].

H3Me3K36 decreases in the setting of either increased or decreased glucose [3]. One possible explanation is that H3Me3K36 is not sensitive to glucose levels themselves but rather other alterations seen in the setting of both maternal hyperglycemia and IUGR, including acidosis and hypoxia. Maternal hyperglycemia leads to fetal acidosis and hypoxia resulting from the increased metabolic activity associated with excess nutrient delivery [18]. IUGR leads to fetal acidosis and hypoxia through decreased nutrient and oxygen delivery from UPI.

Decreased H3Me3K36 of the IGF-1 gene is consistent with the finding of decreased IGF-1 mRNA variant levels in males. Large males from hyperglycemic dams subsequently do not gain weight as quickly and have lower glucose levels by 2 months of age compared to their control counterparts [10], a finding which is *preceded* by decreased IGF-1 mRNA levels in male offspring. This finding suggests decreased male IGF-1 mRNA levels as one mechanism through which large male offspring develop insulin resistance and postnatal growth restriction.

Of note, although maternal hyperglycemia decreases hepatic H3Me3K36 in males, few gender differences were seen in the CON offspring. For example, histone acetylation, also a marker for gene activation [19], of IGF-1 P2 is increased relative to P1 beyond the neonatal period in both genders, a finding seen in other studies [3, 20]. Increased P2 histone acetylation beyond the neonatal period may be an effect of growth hormone signaling [21, 22]. The similarity in histone acetylation and methylation markers and mRNA variant levels occurred in control animals despite housing the animals in different locations and utilizing different control interventions. Similarities that exist in the control animal histone code along IGF-1 reinforced the normal pattern of H3 acetylation and methylation along the IGF-1 gene.

DNA methylation of IGF-1 promoters 1 and 2 remains unchanged in males and is increased in females in this study. DNA methylation is a factor in driving histone code changes [4, 5]. DNA methylation may also aid in nucleosome positioning and thereby effect mRNA transcription [5]. Nucleosomes are approximately 150 base pairs in length, and we found an approximately 150 base pair region of P1 that had less methylation than either 5′ or 3′ of this region in both genders. Thus the increase in DNA methylation in female DOL 21 OHM may contribute to decreased female IGF-1 mRNA variant levels that did not reach statistical significance.

Maternal hyperglycemia did not change serum IGF-1 levels in DOL 21 OHM. IGF-1 serum levels are still present in the setting of decreased or absent hepatic IGF-1 mRNA production, such as the IGF-1 mouse knockout model [2]. The ability of the IGF-1 knockout to maintain serum IGF-1 levels can be achieved through a variety of IGF-1 binding proteins which hold IGF-1 within the serum and may contribute to the normal serum IGF-1 levels in our study [2]. Further, IGF-1 often acts through a paracrine fashion, and thus the importance of decreased IGF-1 mRNA variant levels is in the ability to fine tune IGF-1 expression and may not be reflected completely in serum IGF-1 levels.

One limitation of this study is that maternal hyperglycemia was induced by STZ, and it is possible that STZ crosses the placenta and thus affects fetal and postnatal glucose levels, hepatic IGF-1 mRNA variant levels, and epigenetic characteristics. However, maternal hyperglycemia did not increase glucose levels at DOL 21, indicating that few, if any, offspring pancreatic beta cells were affected directly by STZ. It is also unlikely that STZ directly affected hepatic IGF-1 mRNA variant levels and epigenetic characteristics because STZ is not known to effect the hepatocyte. A further limitation is the small *n* used in this study. However, despite the small *n* we were able to see significant changes in the epigenetic profile of IGF-1 and are consistent with results seen by Fu et al. in the setting of IUGR [3].

In conclusion, maternal hyperglycemia decreases DOL 21 male rat offspring hepatic IGF-1 mRNA variant levels and H3Me3K36 of the IGF-1 gene. These findings are most intriguing given that IUGR also decreases hepatic IGF-1 mRNA variant levels and H3Me3K36 of the IGF-1 gene, and both maternal hyperglycemia and IUGR increase the risk of the same outcome of adult onset insulin resistance. We speculate that decreased H3Me3K36 of the IGF-1 gene in both maternal hyperglycemia and IUGR leads to decreased IGF-1 mRNA variant levels and contributes to the development of adult onset insulin resistance.

Abbreviations

IGF-1: Insulin like growth factor 1
IUGR: Intrauterine growth restriction
OHM: Offspring of hyperglycemic mothers
DOL: Day of life
ChIP: Chromatin immunoprecipitation
H3Me3K36: Histone 3 trimethylation of lysine 36
H3AcK14: Histone 3 acetylation of lysine 14
H3Me2K4: Histone 3 dimethylation of lysine 4
H3Me3K4: Histone 3 trimethylation of lysine 4
UTR: Untranslated region.

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Research Article

The Effect of Neonatal Leptin Antagonism in Male Rat Offspring Is Dependent upon the Interaction between Prior Maternal Nutritional Status and Post-Weaning Diet

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Epidemiological and experimental studies report associations between overweight mothers and increased obesity risk in offspring. It is unclear whether neonatal leptin regulation mediates this association between overweight mothers and offspring obesity. We investigated the effect of neonatal treatment with a leptin antagonist (LA) on growth and metabolism in offspring of mothers fed either a control or a high fat diet. Wistar rats were fed either a control (CON) or a high fat diet (MHF) during pregnancy and lactation. Male CON and MHF neonates received either saline (S) or a rat-specific pegylated LA on days 3, 5, and 7. Offspring were weaned onto either a control or a high fat (hf) diet. At day 100, body composition, blood glucose, β-hydroxybutyrate and plasma leptin and insulin were determined. In CON and MHF offspring, LA increased neonatal bodyweights compared to saline-treated offspring and was more pronounced in MHF offspring. In the post-weaning period, neonatal LA treatment decreased hf diet-induced weight gain but only in CON offspring. LA treatment induced changes in body length, fat mass, body temperature, and bone composition. Neonatal LA treatment can therefore exert effects on growth and metabolism in adulthood but is dependent upon interactions between maternal and post-weaning nutrition.

1. Introduction

Obesity and metabolic-related disorders are considered major health issues worldwide. Over the last decade, the incidence of obesity and overweight has almost doubled in developed countries and the trend is mirrored in developing nations that are transitioning to first-world economies [1]. Obesity results from an interaction of many factors including genetic, physiologic, behavioural, and environmental influences. However, the rapid increases in the rates of obesity suggest that environmental and behavioural influences, rather than genetic causes, are fuelling the present epidemic.

Increasing evidence from both clinical and animal studies has highlighted the link between altered maternal nutrition and the risk of offspring developing obesity and the metabolic syndrome [2–5]. Initial epidemiological studies suggested that fetal growth restriction is correlated with later disease, implying that fetal nutritional deprivation may be a strong stimulus for developmental programming. However, although maternal nutrient deprivation has been well characterized in this context, in many societies, maternal and postnatal nutrition can be excessive. As a result, excessive weight gain and/or obesity are common nutritional problems complicating pregnancy in developed countries. As such, there is now accumulating evidence from human [6] and animal studies suggesting that excess maternal caloric intake has adverse effects on the health and well-being of offspring, independent of postnatal diet [7] and exerted transgenerational effects [8].

Maternal obesity has many adverse outcomes, including labour and delivery complications, fetal and neonatal death, maternal hypertension, and preeclampsia and gestational diabetes [9–12]. In addition to acute risks to the obese mother, negative outcomes extend to offspring, including obesity and cardiovascular disease in adulthood [13–16]. On one hand, while a shared postnatal environment and genetic susceptibility are likely contributors [17], maternal BMI is reported in some cohorts as having greater influence on

offspring than paternal BMI, highlighting the independent influence of the intrauterine environment on offspring adiposity [18].

In a rodent model, we recently reported that offspring born to high-fat-fed mothers showed an obese phenotype in adulthood characterised by hyperinsulinemia and hyperleptinemia, independent of postnatal diet [7, 19]. Although the mechanistic drivers underlying this obesogenic phenotype are still unclear, experimental data in rodents suggest that leptin plays a critical role in underpinning early life influences on postnatal phenotypic development [20–22]. Early studies demonstrated that leptin, an adipokine produced primarily by adipocytes, plays a key role in regulation of energy homeostasis and food intake via its action on specific hypothalamic nuclei [23]. Leptin has been since demonstrated to exert other important functions, including its regulation of bone growth, skeletal metabolism, and linear growth via direct effects on osteoblast and osteocalcin release and growth hormone secretion, respectively [24, 25].

In rodents, a characteristic of the neonatal period is a leptin surge, which normally peaks in the second week of neonatal life [26]. It has been shown that early life nutritional insults affect this surge resulting in altered hypothalamic development [27, 28]. Maternal undernutrition in rats has been shown to result in a blunted and altered timing of the leptin surge in neonatal pups [27], and leptin administration during the neonatal period in *ob*/*ob* mice normalised hypothalamic development and partially normalised orexigenic behaviour [21]. Importantly, postweaning leptin administration had no effect [27], emphasising that, in the rat, the critical stage of hypothalamic leptin regulation is during the first 2 weeks of neonatal life. Our group has previously shown that neonatal leptin treatment can reverse the deleterious effects of maternal undernutrition on postnatal outcomes in male and female offspring [29, 30]. In contrast, Kirk et al. reported that maternal high-fat diet led to an amplified leptin surge in neonatal pups during the first 2 weeks of life, resulting in altered hypothalamic regulation of food intake [31].

There is growing evidence that leptin plays a significant role in the development of an obesogenic phenotype after early-life exposure to an imprudent diet. Despite this, no studies have investigated whether maternal high-fat-diet-induced changes in neonatal leptin action regulate this association. We, therefore, hypothesized that leptin blockade, using a specific leptin antagonist, during the critical neonatal period of leptin sensitivity would ameliorate maternal high-fat-induced obesogenic effects on offspring [7]. We investigated the effect of leptin antagonist administration during the time of the critical neonatal leptin surge on weight gain, food intake, and body composition in male offspring born to mothers fed either a control or a high-fat diet.

2. Methods

2.1. Animal Model. The animal model of maternal high-fat nutrition has been described in detail previously [7, 19]. Briefly, female Wistar rats were time-mated using a rat estrus cycle monitor (EC40, Fine Science Tools, Foster City, CA, USA) to assess the stage of estrus of the animal before introducing the male. Upon confirmation of mating, rats were randomly assigned to one of two maternal diets: the control chow diet throughout pregnancy and lactation (CON, $n = 11$ litters, Diet 2018, Harlan-Teklad, Oxon, UK) or a high-fat diet (MHF, $n = 13$ litters, 45% kcals from fat, D12451, Research Diets, New Brunswick, NJ, USA) to be fed *ad libitum* throughout pregnancy and lactation. Females were housed individually, with free access to water, and bodyweight and food intakes were measured every two days until the end of lactation. At birth, pups were weighed, and on postnatal day 2, litter size was adjusted to 8 pups per litter to ensure adequate and standardized nutrition until weaning. At postnatal day 3, MHF and CON litters were randomly assigned to receive either saline (S) or pegylated rat leptin antagonist (LA, mutant L39A/D40A/F41A, Protein Laboratories Rehovot, Israel). The LA or saline was administrated by subcutaneous injection at postnatal days 3, 5, and 7 at a dose of 12.5 μg/g. Dosage and timing of LA administration was derived from calculated half-life (approximately 20 hours) and prior cited publications [32]. Male pups were weighed daily during the treatment period and then every 2 days thereafter until weaning (P22). At weaning, saline- and LA-treated CON and MHF male offspring were housed two per cage, and randomly placed on either the control rat chow (c) or high-fat (hf) diet until the completion of the trial (day 110). A schematic of the study design is shown in Figure 1.

Body weights and caloric intakes were recorded in offspring every 3 days until the end of the study. Body composition (fat mass, bone mineral content (BMC), and bone mineral density (BMD)) was measured by dual-energy X-ray absorptiometry (DEXA) at P100 under light isoflurane (2%) anaesthesia and using a dedicated small animal software package (Lunar Hologic, Waltham, MA). Rats were culled at P110 by decapitation following anaesthesia with sodium pentobarbitone (60 mg/kg). A tail blood sample was taken for fasting glucose and β-hydroxybutyrate (BHB) measurements (Roche Accucheck) and a rectal temperature measurement recorded. Trunk blood was collected into heparinised vacutainers, centrifuged and plasma stored at -20°C for later analysis. All animal work was approved by the Animal Ethics Committee of the University of Auckland.

2.2. Plasma Analyses. Plasma leptin and insulin concentrations were analysed using commercially available rat-specific ELISAs (no. 900040 and no. 90060, resp., CrystalChem, IL, USA).

2.3. Statistical Analysis. Data were analysed using JMP 7 (SAS Institute Inc., Cary, NC, USA) and R software (v.2.9.0, R Foundation for Statistical Computing, Vienna, Austria) for Windows. All data are presented as mean ± SEM unless otherwise stated. All models were statistically validated for assumptions of normality of residuals and absence of heteroscedasticity. Nonnormal data were log transformed to normalize where necessary. Maternal pregnancy data and neonatal data at birth were analysed using one-way ANOVA.

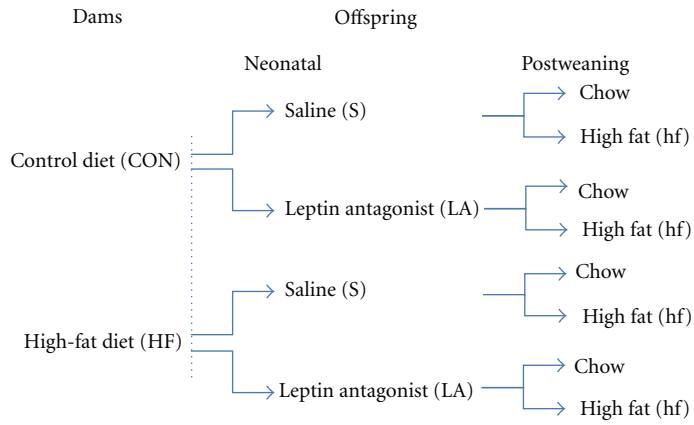

Figure 1: Schematic showing experimental design. There are two levels of maternal nutrition, 2 levels of neonatal treatment, and 2 levels of postweaning diet resulting in a total of 8 experimental groups in a fully balanced $2 \times 2 \times 2$ design.

Maternal caloric intakes; weight and body composition during lactation was analysed by two-way ANOVA with maternal diet and treatment group as factors and litter as covariate. Neonatal and postweaning growth was analysed by repeated measures factorial ANOVA. Although the growth analysis was performed on absolute body weight data, growth figures are shown as relative changes for sake of clarity given the number of experimental groups involved. Preweaning data for pups was analysed by two-way factorial ANOVA with maternal diet and LA administration as factors, and their interactions. Data from adult offspring were analyzed by three-way factorial ANOVA with maternal diet, postweaning diet, and LA treatment as factors, and the interaction between these factors (litter included as a covariate). Post hoc multiple pairwise comparisons were performed using Tukey test. Level of significance was set at $P < 0.05$.

3. Results

3.1. Maternal Data. Consistent with our previous observations [7], maternal HF diet during pregnancy and lactation resulted in a transient increase in caloric intake from day 2 ($P < 0.001$) to day 15 of gestation when intakes returned to levels similar to those observed in CON dams (data not shown). Increased caloric intake in MHF dams was reflected in an increased maternal weight gain by gestational day 7 ($P = 0.04$, Figure 2), which persisted until birth. MHF dams remained heavier than controls from the early neonatal period until mid-lactation when body weights returned to match those of controls. There was no overall significant effect of neonatal LA administration on weight or caloric intake of dams during pregnancy and lactation. At weaning, despite a similar maternal body weight, total fat mass (%) and fat : lean ratios (F/L) were significantly increased in MHF dams compared to CON (% fat: CON 14.7 ± 1.7% versus MHF 22.9 ± 2.3, $P = 0.01$; F/L: CON 0.18 ± 0.08 versus MHF 0.31 ± 0.16, $P = 0.01$). There was no significant effect of neonatal LA treatment or an interaction between treatment and maternal diet, on body composition of dams.

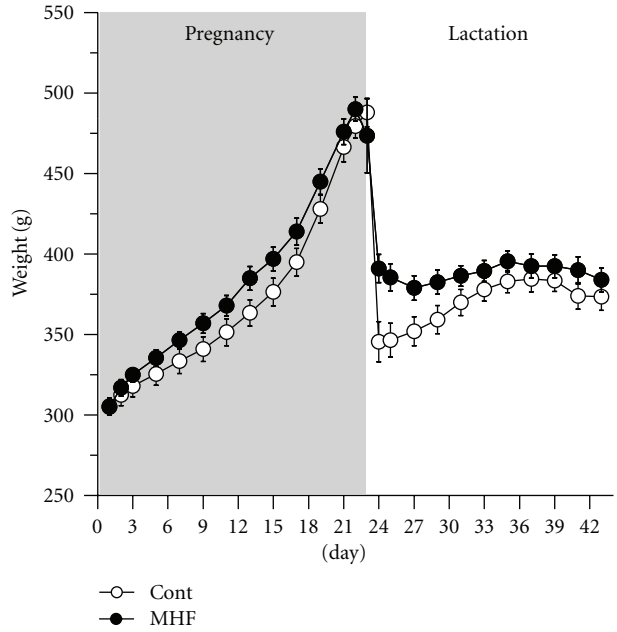

Figure 2: Maternal body weights during pregnancy and lactation. There was no overall significant effect of neonatal LA administration on weight of dams during pregnancy and lactation thus data for Sal and LA groups for each respective maternal diet have been combined. Data are means ± SEM with a minimum of 5 litters per group.

3.2. Neonatal Growth. Birthweights were slightly but significantly reduced in male offspring of MHF dams compared to CON (CON 6.2 ± 0.1 g; MHF 5.9 ± 0.1 g, $P < 0.001$). At P3 and prior to start of LA administration, pups born to MHF mothers remained lighter than CON neonates (CON 7.1 ± 0.1 g; MHF 6.8 ± 0.1 g, $P < 0.05$).

LA administration leads to an increased neonatal weight gain in CON and MHF offspring compared to their saline treated counterparts ($P < 0.005$, Figure 3). The increased weight gain in LA-treated neonates was more pronounced in

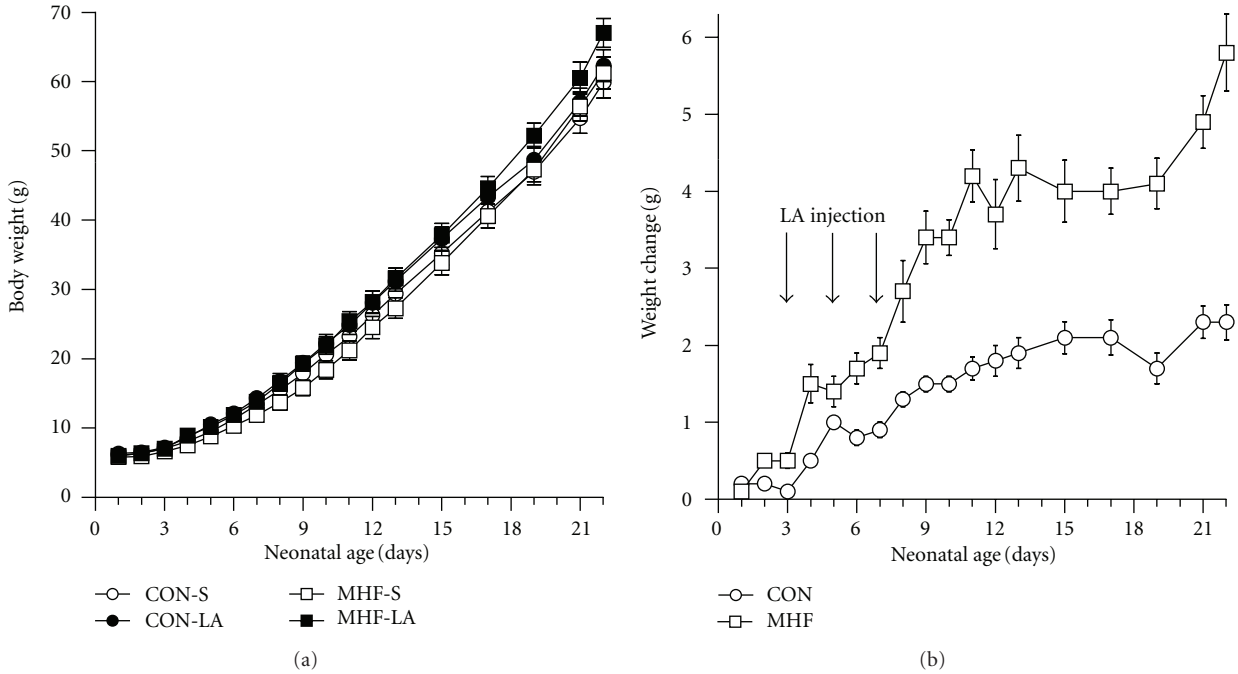

Figure 3: (a) Absolute body weights in male offspring from birth until weaning and (b) changes in neonatal body weights (grams, shown as delta difference in body weight between LA and Sal treated offspring, ΔLA-Sal) in CON and MHF neonates from birth until the time of weaning (day 22). Arrows indicate time of injection with LA at neonatal days 3, 5, and 7. $P < 0.005$ for effect of LA treatment; maternal diet × LA treatment interaction $P < 0.05$. N = minimum 16 per group.

offspring of MHF dams reflected in a maternal diet × LA treatment interaction ($P < 0.005$). By weaning (P22), MHF offspring were slightly but significantly heavier than CON offspring and LA treatment further increased weaning weights in CONT and MHF offspring (P22: CON-S 59.9 ± 0.9 g, CON-LA 62.0 ± 1.1 g, MHF-S 61.8 ± 1.7 g, MHF-LA 66.3 ± 1.1 g, $P < 0.05$ for effect of maternal diet and LA treatment, no interactions).

3.3. Postweaning Growth and Body Composition. An MHF diet had no significant effect on adult body weight at postnatal day 110 but resulted in significantly increased total percent body fat and decreased lean body weight percentage compared to CON animals as quantified by DEXA scanning (Table 1). A postweaning hf diet increased body weight and total body fat mass in all hf-fed groups. Neonatal LA treatment had a significant overall effect on reducing total percent body fat mass, increasing lean mass and a decreased fat : lean ratio (Table 1). A significant maternal diet × LA treatment × postnatal diet interaction ($P < 0.001$) revealed that body weights were significantly reduced in offspring of CON dams that were treated as neonates with LA and fed a postweaning hf diet as compared to saline treated CON offspring fed the hf diet (Figure 4(c)). LA treatment in CON neonates reduced hf diet-induced obesity by approximately 10% and equated to an absolute bodyweight difference of 72.6 g (Figure 4(a)). DEXA analysis of body fat content showed that this reduction in body fat was paralleled by a reduction in fat mass in these animals compared to saline treated (Figure 4(d)). This effect was not observed in MHF offspring where neonatal LA failed to significantly impact on postweaning hf-induced changes in final bodyweight or fat mass (Figures 4(b) and 4(d) and Table 1). This may reflect a more marked increase in relative lean mass in LA-treated CON-hf offspring as compared to LA treated MHF-hf offspring compared to relative saline-treated groups (Table 1).

There were no significant effects of MHF diet, neonatal LA treatment, or postweaning hf diet on total caloric intake (expressed as kcals consumed per gram body weight) across any of the treatment groups (Figures 5(a) and 5(b)).

There were significant overall effects of maternal diet and LA treatment on nose-anus (NA) length (Table 1). Post hoc analysis revealed LA treatment increased NA length in MHF offspring but not CON offspring as reflected in a maternal diet × LA treatment interaction ($P < 0.05$). A postweaning hf diet increased NA length only in MHF offspring (Table 1).

Maternal diet had no overall effect on nose-tail (NT) length (Table 1). There were overall significant effects of neonatal LA treatment and postweaning hf diet on increasing NT length. A significant maternal diet × LA treatment interaction revealed that increases in NT length as a result of neonatal LA treatment were greater in MHF offspring compared to CON offspring for both chow and postweaning hf diets (Table 1).

3.4. Bone Mineral Density (BMD). There was no effect of MHF diet on BMD (Table 1). Neonatal LA treatment reduced BMD in all treatment groups and a postweaning

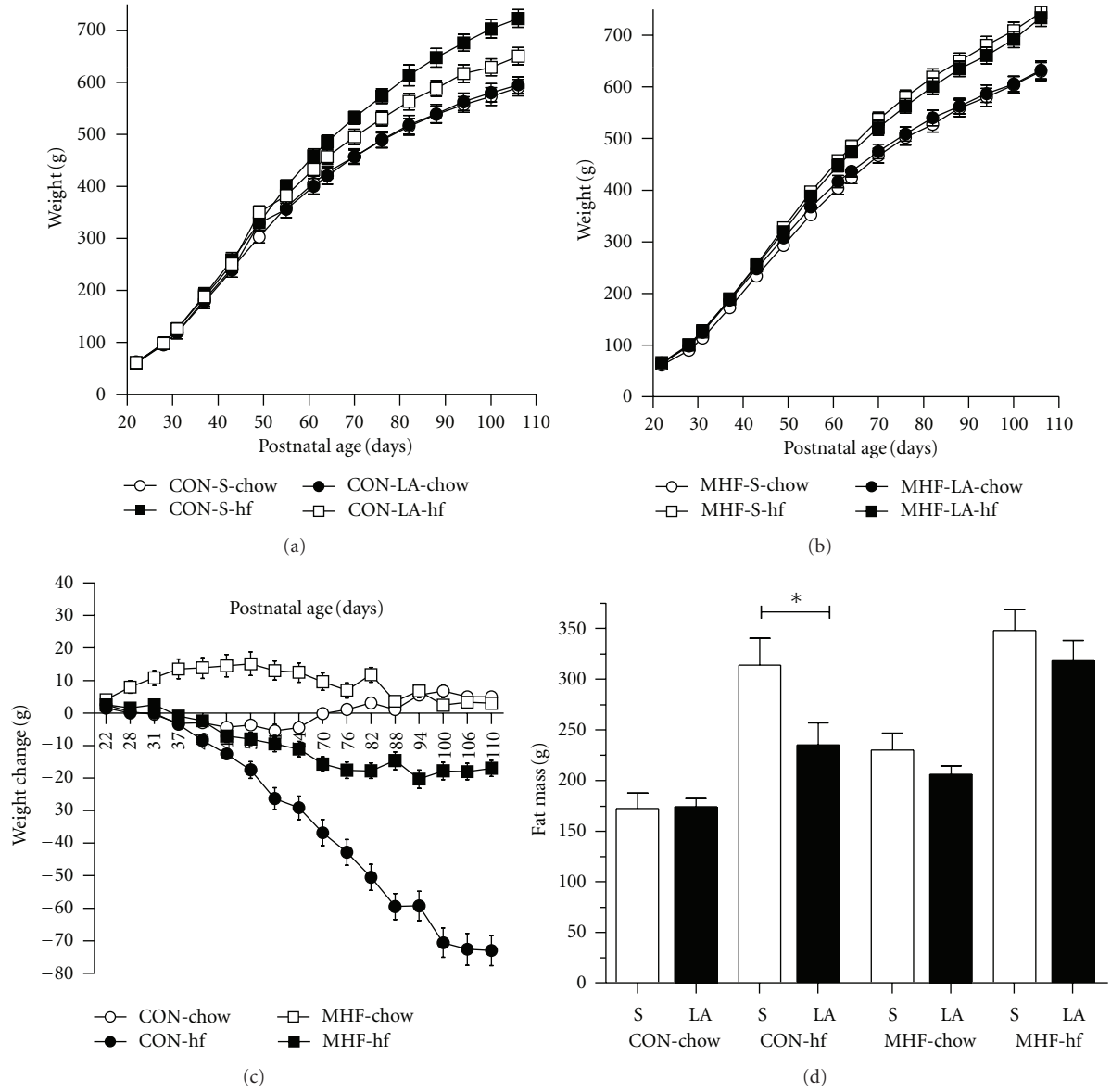

Figure 4: Body weights in male offspring of either CON (a) or MHF (b) mothers treated with either saline or LA as neonates and fed either a chow or hf diet after weaning. Data are means ± SEM, n = minimum 8 per group; (c) change in body weight (grams, shown as delta difference in body weight between LA and Sal-treated offspring, ΔLA-Sal) in CON and MHF offspring fed either a control chow or hf diet from weaning until postnatal day 110. Data are means ± SEM, n = minimum 8 per group. Maternal diet × LA treatment interaction $P < 0.005$; (d) fat mass (g) as quantified by DEXA scanning in offspring of CON of MHF dams, treated with either Sal or LA as neonates and fed either a control chow or hf diet after weaning. Data are means ± SEM, minimum of 8 per group. $^*P < 0.05$.

hf diet increased BMD in all offspring. There were no statistically significant interactions.

3.5. Bone Mineral Content (BMC). An MHF diet had the overall effect of increasing BMC in all offspring (Table 1). Neonatal LA treatment reduced BMC in all treated groups and a postweaning hf diet increased BMC in all offspring. There were no statistically significant interactions.

3.6. Rectal Temperature (RT). An MHF diet significantly increased RT in all MHF offspring (Table 2). Neonatal LA treatment reduced RT in all treatment groups. A postweaning hf diet had no significant effect on RT ($P = 0.092$). There were no statistically significant interactions.

3.7. Leptin, Insulin, Glucose, and β-Hydroxybutyrate (BHB) Levels. An MHF diet significantly increased plasma leptin levels in all MHF offspring (Table 2). Neonatal LA treatment had no overall effect on plasma leptin levels. A postweaning hf diet increases plasma leptin in all hf-fed offspring. There were no statistically significant interactions for plasma leptin. There was a strong

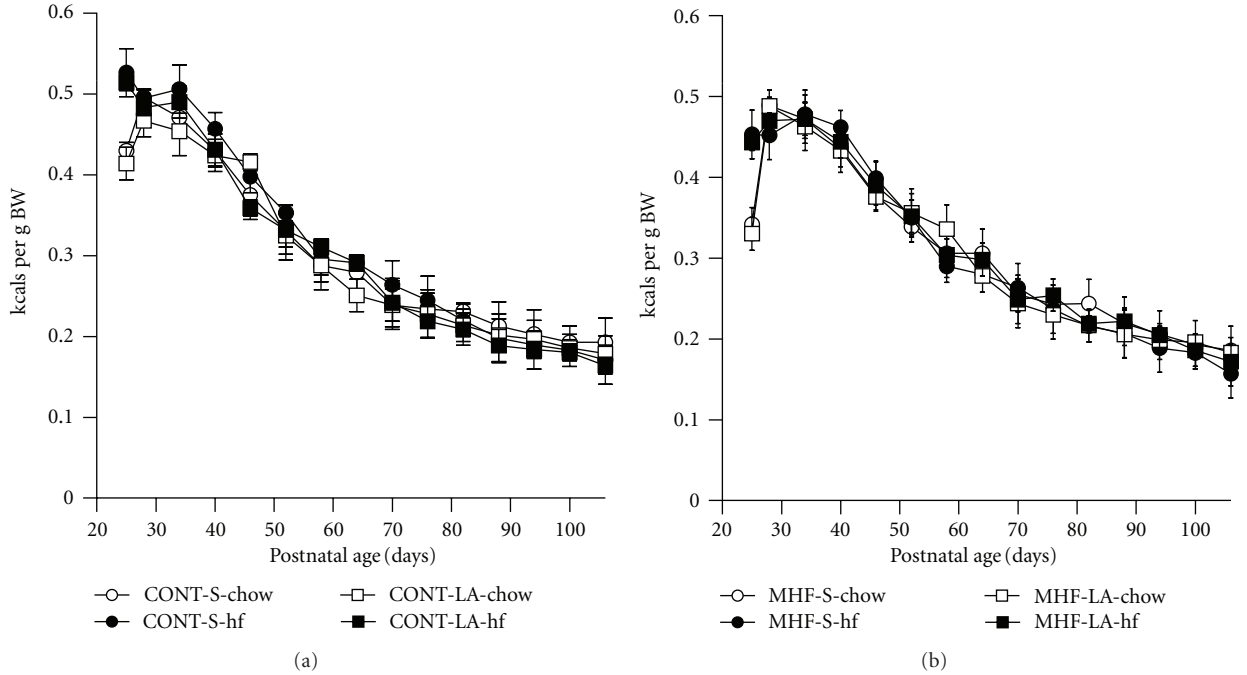

Figure 5: Total caloric intake (kcals consumed per gram body weight) in male offspring of CON mothers (a) or MHF mothers (b) from birth until the completion of the trial. Data are average caloric intakes per cage of 2 animals, minimum of 4 cages per group.

Table 1: Adult (P110) body weight, total fat (%), body length, bone density, and mineral content in CON or MHF offspring treated with either saline (S) or pegylated leptin antagonist (LA) as neonates and fed either a chow or hf diet after weaning. Data are means ± SEM, $n = 8$–16 per group.

Group	Final body weight (g)	Total body Fat (%)	Lean mass (%)	Fat : lean ratio	Nose-anus (mm)	Nose-tail (mm)	BMD (g/cm^2)	BMC (g)
CON-S-chow	590 ± 10	31.0 ± 2.3	67.2 ± 2.2	0.47 ± 0.05	277 ± 2	478 ± 2	0.174 ± 0.001	14.8 ± 0.4
CON-S-hf	723 ± 19	46.6 ± 2.6	52.0 ± 2.5	0.91± 0.09	279 ± 2	484 ± 4	0.183 ± 0.003	17.6 ± 0.6
CON-LA-chow	595 ± 17	29.1 ± 1.1	69.3 ± 0.9	0.43 ± 0.02	277 ± 3	479 ± 4	0.171 ± 0.002	14.5 ± 0.2
CON-LA-hf	650 ± 21	37.9 ± 3.1	60.0 ± 3.8	0.72 ± 0.09	278 ± 2	489 ± 3	0.174 ± 0.003	15.8 ± 0.6
MHF-S-chow	621 ± 11	38.9 ± 2.1	59.6 ± 2.1	0.65 ± 0.06	277 ± 2	477 ± 3	0.176 ± 0.002	15.7 ± 0.3
MHF-S-hf	729 ± 18	51.0 ± 1.6	47.8 ± 1.5	1.06 ± 0.07	280 ± 2	480 ± 3	0.187 ± 0.003	18.7 ± 0.4
MHF-LA-chow	624 ± 13	34.9 ± 1.5	63.5 ± 1.5	0.54 ± 0.03	283 ± 1	490 ± 2	0.171 ± 0.001	14.8 ± 0.3
MHF-LA-hf	710 ± 18	44.1 ± 3.0	50.8 ± 1.9	0.94 ± 0.08	287 ± 2	493 ± 4	0.179 ± 0.002	17.5 ± 0.4
Main effects								
Maternal diet (MD)	$P < 0.05$	$P < 0.005$	$P < 0.0001$	$P < 0.005$	$P < 0.05$	NS	NS	$P < 0.005$
LA treatment (LA)	NS	$P < 0.005$	$P < 0.05$	$P < 0.05$	$P < 0.05$	$P < 0.005$	$P < 0.0001$	$P < 0.005$
PW-diet	$P < 0.0001$	$P < 0.0001$	$P < 0.0001$	$P < 0.0001$	NS	$P < 0.05$	$P < 0.0001$	$P < 0.0001$
Interactions								
MD × LA	NS	NS	NS	NS	$P < 0.05$	$P < 0.05$	NS	NS
LA × PW-diet	$P = 0.05$	NS	NS	NS	NS	NS	NS	NS
MD × PW diet	NS	NS	NS	NS	NS	NS	NS	NS
MD × PW diet × LA	NS	NS	NS	NS	NS	NS	NS	NS

trend toward increased fasting plasma insulin levels in MHF offspring, but this difference did not reach statistical significance ($P = 0.068$). LA treatment had no effect on plasma insulin levels. A postweaning hf diet increased plasma insulin levels in all hf-fed groups (Table 2). Fasting plasma glucose levels were similar between the groups (Table 2). Blood BHB levels were not altered by MHF diet or neonatal LA treatment but were significantly increased in all hf-fed offspring compared to chow-fed offspring (Table 2).

Table 2: Fasting plasma leptin, insulin, glucose, and β-hydroxybutyrate (BHB) measurements and rectal temperature in CON and MHF offspring treated with either saline (S) or pegylated leptin antagonist (LA) as neonates and fed either a chow or hf diet after weaning. Data are means ± SEM, $n = 8$–16 per group, no significant statistical interactions.

	Leptin (ng/mL)	Insulin (ng/mL)	Glucose (mmol/L)	BHB (mmol/L)	Rectal Temperature (°C)
CON-S-chow	10.4 ± 0.8	2.9 ± 0.2	5.98 ± 0.26	1.07 ± 0.07	37.19 ± 0.18
CON-S-hf	34.1 ± 3.6	4.6 ± 0.4	6.06 ± 0.32	1.57 ± 0.14	37.14 ± 0.13
CON-LA-chow	11.2 ± 1.4	3.4 ± 0.6	6.07 ± 0.37	1.24 ± 0.14	36.63 ± 0.19
CON-LA-hf	24.4 ± 3.9	5.9 ± 0.7	5.91 ± 0.27	1.29 ± 0.16	37.08 ± 0.14
MHF-S-chow	20.9 ± 2.9	4.4 ± 0.9	5.96 ± 0.26	0.92 ± 0.08	37.32 ± 0.15
MHF-S-hf	36.1 ± 3.0	6.1 ± 0.7	5.82 ± 0.32	1.47 ± 0.10	37.63 ± 0.21
MHF-LA-chow	16.7 ± 2.8	4.0 ± 0.6	5.28 ± 0.34	1.05 ± 0.14	37.08 ± 0.19
MHF-LA-hf	31.8 ± 4.2	6.1 ± 0.9	5.24 ± 0.31	1.61 ± 0.14	37.28 ± 0.11
Main effects					
Maternal diet	$P < 0.05$	$P < 0.05$	NS	NS	$P < 0.05$
LA treatment	NS	NS	NS	NS	$P < 0.05$
PW-diet	$P < 0.0001$	$P < 0.0001$	NS	$P < 0.0001$	NS

4. Discussion

These results have demonstrated for the first time that early-life manipulation of the leptin axis via neonatal leptin antagonism can exert marked effects on growth and body composition, which are dependent upon prior maternal nutrition status and postweaning diet. Investigators using neonatal leptin treatment given to offspring of normally fed dams have shown increased adiposity and leptin and insulin resistance in offspring in later life [33–35]. The present result shows that the reverse can hold true. Control offspring, given a leptin antagonist prior to being fed an obesogenic hf diet postweaning, show an amelioration of a diet-induced fat accumulation and reduced linear body growth. The marked contrast in adult phenotype in offspring of normally nourished mothers, based on exposure to either leptin or leptin antagonism during early-life development, further serves to highlight how critical the maintenance of leptin threshold levels is during this period of developmental plasticity.

As we have shown previously [7], maternal high-fat nutrition resulted in increased adiposity, leptin, and insulin concentrations in offspring compared to offspring of control mothers, independent of postweaning diet. There is a well-characterized leptin surge in the first two weeks of life in the rodent [26] although the source of the leptin is yet to be defined with the surge occurring independently of changes in neonatal body weight trajectory and milk leptin intake [31]. We have previously shown that offspring of MHF mothers are hypoleptinemic at birth [7]. This concurs with the increased sensitivity to body weight gain in MHF neonates treated with the LA as compared to CON offspring. In other studies, hypoleptinemic offspring of mothers undernourished during pregnancy have either a delayed [36] or premature leptin surge [27]. However, there is little known about the leptin surge in models of maternal obesity. Kirk et al. recently reported that rat offspring of mothers fed an obesogenic diet had normal serum leptin levels at birth but displayed an amplified and prolonged neonatal leptin surge, which was accompanied by an elevation in leptin mRNA expression in abdominal white adipose tissue [31]. However, it is unknown whether the leptin surge in the MHF offspring of the present study is altered.

Although inborn leptin deficiency causes weight gain, it is unclear whether induced leptin deficiency in adult wild-type animals would be orexigenic. Leptin antagonists have only recently become commercially available and provide an invaluable tool for investigating central and peripheral leptin deficiency and exploring the involvement of leptin in metabolic processes. Previous reports using a nonpegylated leptin antagonist have been problematic. The extremely short half-life of the antagonist necessitated administration of supraphysiological doses to induce a clinical response and was not sufficient to induce a true metabolic state of leptin deficiency [32, 37]. Hormones with molecular masses similar to that of leptin are cleared primarily via the kidney with a half life of only 8–30 minutes [38].

The effect of early postnatal leptin blockade in normal rat neonates has previously been reported in the study by Attig et al. [39]. In this work, the authors studied the long-term effect of neonatal therapy with a non-pegylated leptin antagonist (day 2 to day 13) in female Wistar rats [39]. In contrast to the present study, they showed that leptin antagonism induced a decrease in neonatal weight gain, which has previously been commonly associated with neonatal leptin treatment [30, 40]. Later in life, the leptin disruption led to a higher sensitivity to diet-induced obesity, as shown by a higher body weight gain when challenged with a high-energy diet, associated with increased adiposity and leptinemia. These animals also displayed a phenotype of leptin resistance at 4 months, characterized by the inability of treated animals to respond to leptin by failing to reduce food intake and showing reduced birth weight. Overall, the long-term effect in the Attig study was paradoxically similar to that reported for rats treated with leptin during neonatal

life [30, 40]. Importantly, the molecule used as the leptin antagonist was different from the one used in our study. Indeed in this work, the authors used the leptin mutein, a molecule acting as an antagonist, with *in vivo* effects previously validated only using intracerebroventricular, but not subcutaneous, administration [41–44]. Furthermore, this antagonist, obtained by alanine mutagenesis of amino acids 39 to 41-42, has an extremely short half-life and high doses are required to produce a clinical response that is similar to a true metabolic state of leptin resistance.

The present study utilised a recently developed rat-specific pegylated LA whereby the attachment of polyethylene glycol increased the overall molecule size to 70 kDa. Pegylation of the LA results in an approximate 30-fold increase in *in vivo* half-life [32], thus true states of induced leptin deficiency are possible at physiologic doses. To date, only one prior study has examined the effects of the pegylated LA moiety, albeit in normal postweaning animals [37] where it was shown that treatment with the pegylated LA to postweaning mice results in a rapid and dramatic increase in food intake and weight gain [32]. The blood brain barrier (BBB) in the neonatal rat is relatively immature; pegylated leptin antagonist has been shown to block circulating leptin from crossing the BBB, an action that would attenuate the anorexigenic effect of leptin [32]. It is difficult to extrapolate the Elinav et al. study to the present study. The windows of treatment are different, BBB permeability is at different developmental stages (neonatal versus postweaning), and offspring responsiveness to leptin intervention is known to elicit sexually dimorphic responses [29, 30]. In addition, the work in the mouse examined the immediate phenotypic response to LA treatment, whereas the present study examines a postnatal phenotype derived from an early-life neonatal intervention. However, consistent with the reports from mice, the present result demonstrated that LA treatment induced a significant increase in body weight over the neonatal treatment period.

In the present study, neonatal leptin antagonism, despite having significant effects on pre-weaning weights in offspring of MHF mothers, had no effect on postnatal weight gain in CON or MHF offspring fed the standard chow diet. There was, however, a marked effect of neonatal LA treatment in reducing body weight gain in CON offspring fed the hf diet after weaning. Conversely, LA treatment to MHF offspring subsequently fed the hf diet had no significant effect on body weight; independent of changes in body weight and circulating plasma leptin concentrations. LA treatment significantly reduced fat deposit weight in CON but not MHF offspring. Interestingly, neonatal LA treatment did not alter postweaning caloric intake, thus the observed changes in body weight gain are independent of food intake and suggest a lack of effect of LA administration on the arcuate nucleus and related feeding circuitry, as has been reported with neonatal leptin treatment in the *ob/ob* mouse [21].

Nose-anus lengths were increased in MHF offspring but not CON offspring, which may suggest that altered effects on the growth-hormone- (GH-) insulin-like growth factor (IGF) axis are mediated by neonatal LA exposure. The observed change in tail length in CON and MHF LA-treated offspring was unexpected but may have resulted from altered thermoregulatory set-point processes as reflected in the significant differences in basal body temperature. In the rat, a significant portion of total body heat loss occurs through sympathetically mediated changes in tail blood flow [45]. However, since rectal temperature was decreased in LA-treated CON *and* MHF offspring, it is difficult to explain the disparate changes in tail length to thermoregulatory processes and, as with nose-anus length, may reflect LA-induced alterations in the GH-IGF axis in MHF offspring as compared to controls or development of a thrifty metabolic phenotype as regards thermogenesis and energy expenditure. Future independent studies looking at brown fat thermogenesis and uncoupling proteins may further explain this observation.

The effect of LA on bone formation has not previously been described. Bone morphology was significantly altered in adult offspring following neonatal LA treatment with overall significant reductions in BMC and BMD. It is well established that leptin treatment can result in enhanced bone formation and promotion of pro-osteogenic factors in bone marrow [46, 47], and the current data suggests that the reverse holds true for leptin antagonism and further work investigating specific bone markers is now warranted.

This is the first study designed to examine the efficacy of neonatal leptin antagonism following altered maternal nutrition and its interaction with differing levels of postweaning nutrition, on offspring phenotype development. Responsiveness to neonatal leptin antagonism is dependent upon both maternal and postweaning nutrition, with minimal efficacy in chow-fed offspring of either CON or MHF mothers. More studies are now required to further understand the mechanistic underpinnings of the present observations, including characterization of the effects of leptin antagonism on the timing and magnitude of the leptin surge in offspring of mothers with different dietary backgrounds. However, it is important to recognise that leptin-mediated development of feeding circuits occurs postnatally in the rodent and occurs *in utero* in primates, including humans, and thus timing of intervention strategies may be different. Nonetheless, taken together, the data on both neonatal leptin treatment and leptin antagonism in the setting of both normal and nutritionally challenged pregnancies serves to highlight the important role of leptin regulation during critical early-life windows of development on lasting growth and metabolic function in offspring.

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Review Article

The Interplay between Estrogen and Fetal Adrenal Cortex

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Estrogen is a steroid hormone that regulates embryogenesis, cell proliferation and differentiation, organogenesis, the timing of parturition, and fetal imprinting by carrying chemical messages from glands to cells within tissues or organs in the body. During development, placenta is the primary source of estrogen production but estrogen can only be produced if the fetus or the mother supplies dehydroepiandrosterone (DHEA), the estrogen prohormone. Studies show that the fetal zone of the fetal adrenal cortex supplies 60% of DHEA for placental estrogen production, and that placental estrogen in turn modulates the morphological and functional development of the fetal adrenal cortex. As such, in developed countries where humans are exposed daily to environmental estrogens, there is concern that the development of fetal adrenal cortex, and in turn, placental estrogen production may be disrupted. This paper discusses fetal adrenal gland development, how endogenous estrogen regulates the structure and function of the fetal adrenal cortex, and highlights the potential role that early life exposure to environmental estrogens may have on the development and endocrinology of the fetal adrenal cortex.

1. Introduction

The fetal-origin hypothesis put forth by Dr. David Barker nearly three decades ago challenged the traditional views on the pathogenesis of common chronic diseases [1]. The Fetal-Origin Hypothesis, later termed the Developmental Origin of Health and Disease, established the principle that perinatal events are memorized by the developing organism through fetal and neonatal imprinting [2]. In light of this hypothesis, perinatal events can be thought of as the foundation for structural and functional development of an organism. All organisms arise from a unique DNA sequence that gives rise to specialized cell phenotypes through well-regulated gene expression and epigenetic regulation [3]. Each cell phenotype can be influenced by its internal and external environment. In the human body, there are approximately 200 specialized cells that are influenced by internal and external steroid hormones during development [3].

Steroid hormones act as chemical messengers to induce both slow genomic and rapid nongenomic responses and, thus, modulate a wide array of essential cellular and physiological responses. In the initial stages of pregnancy, steroid hormones send signals that allow the embryo to successfully implant in the posterior wall of the uterus and regulate cell proliferation, differentiation, and gene transcription. In the later stages of gestation, steroid hormones support fetal growth and development by modulating metabolic processes of embryogenesis and organogenesis. Steroid hormones also play a pivotal role in regulating the timing of parturition. Different steroid hormones are expressed at different stages of intrauterine life suggesting that temporal and spatial expression of steroid hormones is critical for fetal growth and development [4, 5]. Of all the steroid hormones produced during pregnancy, estrogen has attracted much interest because it is expressed at every stage of gestation and modulates many intrauterine processes.

Estrogen synthesis takes place in the ovaries, adrenal cortex, and the placenta. During pregnancy, the placenta becomes the primary site of estrogen synthesis; however, placental estrogen production can only be achieved through

Table 1: The effects of estrogen on the mother and the fetus.

Estrogen in maternal circulation:
(i) Enhances myometrial activity
(ii) Soften collagen fibers in the cervical tissue
(iii) Promotes myometrial vasodilation
(iv) Increases uterine blood flow
(v) Increases production of insulin-like growth factors (IGF-I/II) and binding proteins
(vi) Promotes growth of the uterus, vagina, and breast
(vii) Increases pituitary secretion of prolactin
(viii) Increases sensitivity of the maternal respiratory center to carbon dioxide
(ix) Stimulates synthesis and turnover of phospholipids
(x) Increases serum binding proteins and fibrinogens to decrease plasma proteins
(xi) Increases the sensitivity of the uterus to progesterone in late pregnancy
(xii) Helps to control behavior including fatique, forgetfulness, poor concentration, as well as mild mood changes including irritability and depressed mood
(xiii) Regulates salt and water retention
Estrogen in fetal circulation:
(i) Helps to maintain chemical levels in the bloodstream to achieve intrauterine homeostasis, which is the state of stability within the body
(ii) Promotes maturation of fetal organs
(iii) Regulates the fetal neuroendocrine system that controls reaction to stress, digestion, immune function, mood and emotion, sexuality, energy storage, and/or expenditure
(iv) Regulates timing of parturition

input from fetal and/or maternal adrenal cortex. This is because of the placenta's inability to produce the androgenic C_{19} steroid (dehydroepiandrosterone, DHEA, and its sulfoconjuate, DHEA-S), the essential substrate for placental estrogen [4, 6, 7]. The fetal adrenal gland provides a larger proportion of the byproducts used for placental estrogen production [8]. Appropriate development and function of the fetal adrenal cortex is therefore critical for placental steroid production (i.e., estrogen, cortisol, and aldosterone), fetal maturation, and perinatal survival [9].

Emerging studies suggest that environmental estrogens can disrupt the natural interplay between estrogen and functional biology of the fetal adrenal cortex [10, 11]. Humans are exposed daily to environmental estrogens through the food supply and the use of pesticides, herbicides, petroleum byproducts, and plastics. Some examples include soy isoflavones, bisphenol A, DDT, polychlorinated biphenyls, polybrominated diphenyl ethers, and a variety of phthalates. Although environmental estrogens pose minimal threat to adults, they may have an adverse effect on human health if exposure occurs during critical stages of development when cells are more easily influenced by steroid hormones [2]. Most of the supporting studies in this area have focused on a synthetic estrogen, diethylstilbestrol (DES). From about 1940–1970, DES was prescribed to pregnant women based on the belief it could prevent miscarriage (it is no longer used because of adverse effects, particularly to offspring). In vitro assays have shown that compared to 17β-estradiol the estrogenic potency of DES is 0.5 and that of all other environmental estrogens listed previously is <0.0001 [12, 13]. Based on the estrogenic potency, the adverse effects induced by DES may be more severe than those induced by environmental estrogens and thus have more subtle effects that are nonetheless disrupting placental estrogen production and fetal development. This paper examines the available literature on fetal adrenal gland development, how endogenous estrogen regulates the structure and function of the fetal adrenal cortex, and highlights the potential role that early life exposure to environmental estrogens may have on the development and endocrinology of the fetal adrenal cortex.

2. Estrogen Synthesis in Utero

Pioneering work by Edward Adelbert Doisy revealed that three forms of estrogen (estradiol, estriol, estrone) are present in the urine of pregnant women [15]. These three forms of estrogen have also been detected in the fetal circulation throughout gestation and in the umbilical cord plasma at birth [16]. Concomitantly, the rate of estrogen production and the amount of estrogen in the maternal and fetal plasma increases markedly throughout pregnancy. By term, estradiol and estrone levels are 100-fold higher than those of nonpregnant women and estriol levels are up to 1000-fold higher [8]. Although these high concentrations of estrogen are destined for maternal compartments, they also induce a number of biological effects on the developing fetus (Table 1).

During early stages of pregnancy, estrogen is synthesized by the corpus luteum in the maternal ovaries and its primary function is to ensure that the mother does not reject the developing embryo. To accomplish this, estrogen enhances myometrial activity, softens collagen fibers in the cervical tissue, and promotes vasodilation of the uterus. Around the 8th week of gestation when the fetoplacental unit has a highly vascularized network, placenta becomes the primary source of estrogen production [4]. However, the production of estrogen does not rely solely on the placenta. The placenta converts C_{19} steroids into estrogen but cannot produce estrogen de novo from acetate or cholesterol and cannot convert pregnenolone or progesterone into C_{19} steroids because it lacks the primary 17α-hydroxylase (P450c17) enzyme [4, 6, 7]. Estrogen synthesis by the primate placenta is therefore achieved through stereogenic interplay between the maternal-placental unit and the fetal-placental unit (Figure 1). In this system, the maternal-placental unit permits low-density lipoproteins (LDLs) and/or free cholesterol to pass through the placenta and enter the fetal circulation. The fetal adrenal cortex, which expresses high amounts of P450c17 enzyme, has a highly developed ability to bind and use cholesterol to produce the DHEA and DHEA-S, which are the essential substrates for placental estrogen production. Near term, 60% of the total DHEA-S used by the placenta for estradiol and estrone synthesis is derived from the fetal

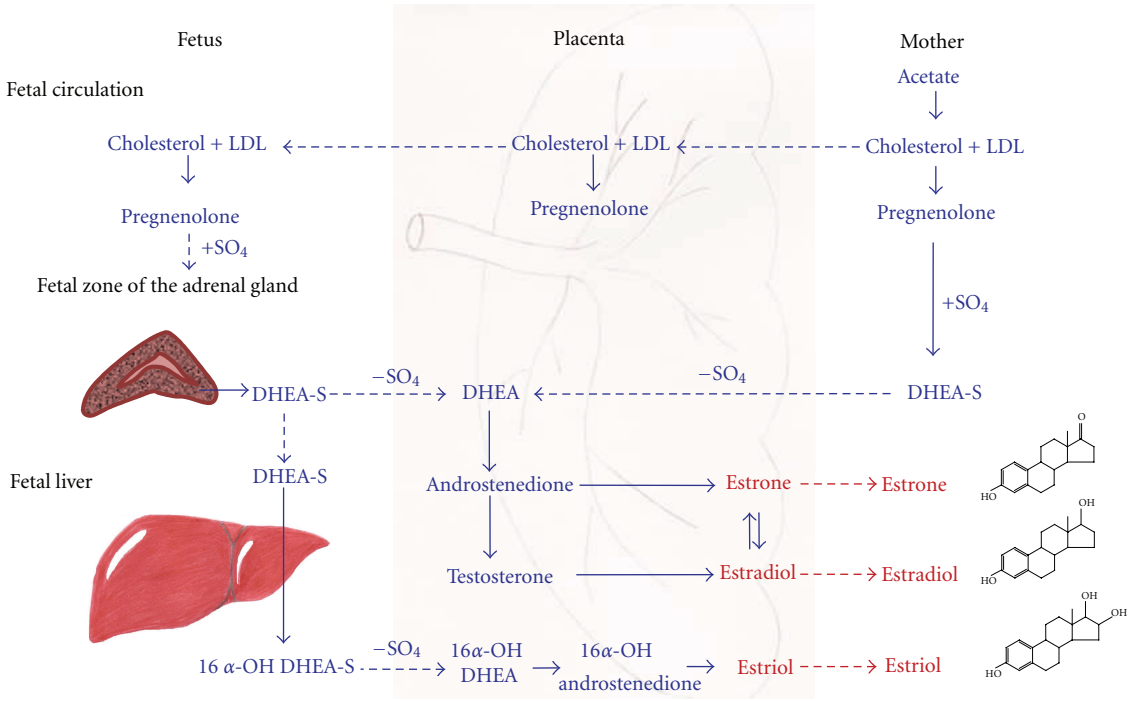

Figure 1: The role of maternal, placental, and fetal units in the biosynthesis of estrone, estradiol, and estriol. (LDL: low-density lipoproteins; DHEA-S: dehydroepiandrosterone sulfate; OH: hydroxyl). This figure has been modified from [8, 14].

adrenal cortex, whereas only 40% is derived from the maternal adrenal cortex [8]. The fetus, unlike the mother, also produces 90% of 16α-hydroxyl DHEA-S, which is a substrate for estriol synthesis. Therefore, the fetus rather than the mother provides most of the byproducts used for placental estrogen production [8].

3. Fetal Adrenal Gland Development

Development of the fetal adrenal gland is a dynamic process that is characterized by rapid cell growth, unique morphological development, and high steroidogenic activity. Throughout gestation, the size and weight of the fetal adrenal gland continuously grow but, unlike most other organs, the adrenal gland regresses substantially from fetal to adult life [17, 18]. At birth, the fetal adrenal gland weighs between 3 and 4 grams and is 20 to 30 times larger than its adult counterpart [17–20]. Moreover, its size at birth is approximately equal to that of the fetal kidney [4]. Studies focusing on structural biology have revealed that the large size of the fetal adrenal gland depends on a single structural compartment called the fetal zone (Figure 2), identified by Elliott and Armour in 1911 [21]. The fetal zone occupies the central region of the fetal adrenal cortex and by mid-gestation accounts for 80–90% of the fetal adrenal gland [17, 22]. Functionally, the fetal zone resembles the adult zona reticulosa because it is the primary site of steroidogenesis [23]. Beginning around the 10th week of gestation, the fetal zone expresses P450c17 enzyme and produces DHEA, the precursor for placental estrogen production. Therefore, fetal zone is the primary site of estrogen synthesis during gestation

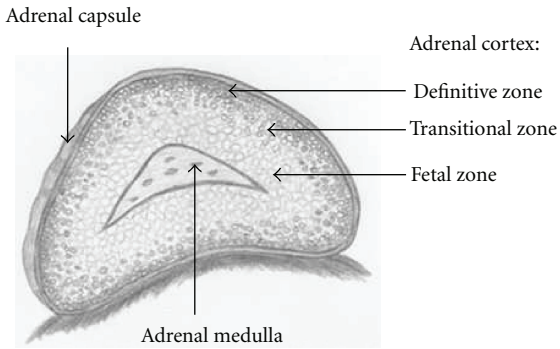

Figure 2: Illustration of the midgestation human fetal adrenal gland.

[4]. Because placental estrogen production is exclusive to intrauterine life, the fetal zone begins to regress at birth and completely disappears in the first few months of life. The demise of the fetal zone induces postnatal remodeling and causes more than a 90% reduction in the adrenal gland size [23].

In addition to the fetal zone, the fetal adrenal cortex in utero is composed of two other morphologically distinct zones: the definitive zone and the transitional zone [9]. The definitive zone, which is also called the neocortex, develops as a narrow band of tightly packed cells surrounding the fetal zone. It expresses the enzyme 3β-hydroxysteroid dehydrogenase (3β-HSD) that produces aldosterone. Thus, it is believed to be the site that gives rise to the postnatal adrenal glomerulosa [23, 24]. The third zone, the transitional zone, develops in late gestation between the fetal and definitive

zone and gives rise to zona fascicularis that produces glucosteroids [24, 25]. The formation and dynamics of the fetal zone, definitive zone, and transition zone form the fetal adrenal cortex. In turn, the adrenal cortex can have long-term effects on the developing organism.

4. Embryogenesis and Development of the Fetal Adrenal Gland

Embryonic development of the fetal adrenal gland involves cellular hyperplasia, hypertrophy, migration, and senescence [4, 19]. As early as five weeks of gestation, the migration of primitive cells accumulates at the cranial end of the mesonephros in what is called the "adrenal blastema" [19]. The adrenal blastema, which is thought to be the first identifiable manifestation of the adrenal gland, exhibits mitotic activity, suggesting that it develops by hyperplasia [4, 19, 26]. Thereafter, neighboring primordial cells which are destined to become steroidogenic cells are recruited to the adrenal blastema [27]. Interestingly, these primordial cells are the same cells that give rise to steroidogenic activity in the gonads [27]. Ultimately the migration of primordial cells from the primordial mesoderm determines whether these cells will commit to the adrenal gland or the gonads. Cells on the dorsal aortal side of the primordial mesoderm give rise to steroidogenic activity in the adrenal cortex, whereas those on the coelomic epithelial side migrate towards the gonads. The cells committed to the adrenal gland form a cord cluster with the adrenal blastema. The cord cluster cells can then either differentiate into large polyhedral cells that give rise to primordium of the fetal zone or can maintain their small morphology and form the definitive zone of the fetal adrenal cortex [4].

The large polyhedral cells have unique characteristics including a larger number of tubular smooth endoplasmic reticula, large Golgi complexes, and a greater number of mitochondria with well-defined cristae. These cells are also characterized by their high lipid production and abundance of highly dense bodies [4]. These cellular properties are consistent with characteristics of steroid-producing cells keeping in agreement that the fetal zone is the primary site of steroidogenesis in the fetal adrenal gland [4]. The cells in the fetal zone aside from having a unique structural composition are arranged in a specific manner that optimizes the production of DHEA-S. The cells in the central portion of the fetal zone are arranged far apart in a netlike pattern with many vascular sinusoids, while the cells on the outer region of the fetal zone are arranged in tightly packed cords [28]. This type of arrangement is designed to speed up the transfer of molecules through the cells and promotes steroidogenesis.

Comparative studies have shown that the cells in the definitive zone are two to five times smaller than those in the fetal zone. They are packed tightly together with small ribosomes, small dense mitochondria containing cristae, and a poor lipid profile [4]. Out of all these cellular characteristics it is the poor lipid profile that has the greatest biological effect as lipid composition modulates steroidogenic activity. Cells with a lipid-rich profile can produce steroidal compounds, whereas those that have a lipid-poor profile lack the fundamental building blocks required for steroidogenesis. The lipid-poor profile in the definitive zone of the adrenal gland is observed only in the first half of pregnancy. At mid-gestation, the definitive zone synthesizes aldosterone, a cholesterol byproduct, and thus, its cellular lipid profile improves around this time [28].

Another unique property of the definitive zone is its mitotic activity. By 10–12 weeks of gestation, the definitive zone exhibits many mitotic subunits [19]. Thus, the definitive zone grows mainly by hyperplasia, whereas the fetal zone grows by hypertrophy [18, 19]. The rapid cell proliferation in the definitive zone causes mitotic pressure in the periphery of the adrenal cortex leading to centripetal migration of cells to the fetal zone [19, 20]. Work by Morley et al. [29] showed that in a mouse embryo the centripetal invasion of cells is only established in the later stages of the fetal life when the lipid composition of cells is improved. This centripetal migration lends support to the migration theory of the adrenal cortical cytogenesis and suggests that the definitive zone is the stem cell compartment from which the inner cortical zones are developed [4]. It is hypothesized that soon after birth, the fetal zone disappears due to increased apoptosis in this region and the centripetal migration of cells from the definitive zone drives the development of zona glomerulosa and fasciculate [19, 30].

The adrenal medulla which is composed of hormone-producing chromaffin cells does not exist as a discrete structure throughout gestation. Instead it is composed of small islands of chromaffin cells that in the first few weeks of gestation are scattered throughout the fetal adrenal cortex but then aggregate in the fetal zone. The disappearance of the fetal zone at the beginning of postnatal life stimulates the already aggregated chromaffin elements around the central vein to form elementary medulla. This elementary medulla undergoes extensive remodeling, and by the fourth week of postnatal life, almost all of the chromaffin cells are clustered in the central region of the adrenal gland. However, it is not until one to two years postpartum that the morphological development of the adrenal medulla resembles its adult counterpart [4]. Further research is needed to determine whether disruption of fetal adrenal cortex has an effect on the development and function of the adrenal medulla.

The development of the fetal adrenal gland is a well-orchestrated phenomenon that requires tight regulation. Significant progress has been made in elucidating the role of steroidogenic enzymes and nuclear receptors, fetal and maternal hypothalamic-adrenal function, and placental estrogens on fetal adrenal function, growth, and development.

5. The Role of Steroidogenic Enzymes and Nuclear Receptors in Differentiation of Cortical Zone Function

The steroidogenic potential of fetal cortical zones depends on its steroidogenic enzymes and nuclear receptors. The two primary steroidogenic enzymes of the fetal adrenal gland are P450c17 and 3β-HSD [4, 23]. Findings from immunohistochemistry have revealed that P450c17 enzyme is expressed in the fetal zone, 3β-HSD is expressed in the definitive zone,

and that both enzymes are expressed in the transition zone [4, 31]. These patterns of enzyme expression provide critical clues regarding the steroidogenic role of each fetal adrenal zone. The P450c17 enzyme stimulates DHEA-S production, the 3β-HSD enzyme drives aldosterone production, and the combined effects of both enzymes are responsible for cortisol synthesis [4]. Based on this evidence and on the in situ patterns of cholesterol cleavage, it has been determined that the fetal zone, definitive zone, and transition zone are sites of DHEA-S, aldosterone, and cortisol synthesis, respectively [4]. For most of human pregnancy, the fetal adrenal cortex lacks the 3β-HSD enzyme inhibiting its cortisol and/or aldosterone production. Therefore, the cholesterol substrate is used primarily for DHEA-S production, making it the primary byproduct of the fetal adrenal gland during gestation [32, 33].

According to human experiments, radiolabeled DHEA-S can be converted to estradiol when perfused through the placenta [34]. Human placenta produces estrogen by the aromatization of DHEA and its 16α-hydroxylated metabolite (16α-OH DHEA) that is produced by the combined efforts of the adrenal cortex and the liver [4, 35]. In the placenta, 3β-HSD converts DHEA to androstenedione. Thereafter, the androstenedione can either be converted to estrone by an aromatase enzyme or to testosterone by 17β-hydroxysteroid dehydrogenase (17β-HSD). The newly synthesized estrone and/or testosterone can be further converted to estradiol by 17β-HSD or aromatase enzyme, respectively (Figure 1).

6. The Relationship between Fetal and Maternal Hypothalamic-Adrenal Function

The ongoing interaction between the fetus and the mother has been the focus of many studies as scientists strive to understand the factors responsible for maintaining pregnancy and stimulating labor and delivery. The essential and universal component of pregnancy is the endocrine communication between the mother and the fetus which greatly depends on the maternal and fetal adrenal cortex. To understand the specific role of fetal and maternal adrenal gland function during gestation, two adrenoectomized models have been developed: the maternal adrenalectomy model and the fetoectomy model. In the first model, which is usually performed in rodents, the adrenal glands are surgically removed from the mother during gestation while the placenta and the fetus are kept in situ. In the second model (i.e., fetoectomy), which is typically performed in primates, the fetus is surgically removed from the uterus and the placenta is kept in situ. The use of these two experimental models has helped scientists uncover some information about the interaction between the maternal and fetal adrenal gland function.

Studies examining maternal adrenalectomy have revealed that the absence of maternal adrenal gland causes a substantial reduction in plasma cortisol and estrogen levels. These endocrine disruptions activate the fetal pituitary gland to secrete adrenocorticotropic hormone (ACTH) [37–39]. Upregulation of ACTH promotes fetal production of corticosteroids (i.e., DHEA and DHEA-S) from the fetal adrenal cortex, which serve as substrates for placental endocrine production (Figure 3). Under these conditions, the fetus is placed under stress and its fetal adrenal cortex takes over at least part of the maternal adrenal function. In a study investigating Sprague-Dawley rats, plasma DHEA-S levels of adrenalectomized dams rose from very low levels at 18 days of gestation to levels that were substantially higher than those of sham rats on 21 days of gestation [40]. This finding suggests that there is a critical factor that compensates for the lack of maternal corticosteroid production. Based on the available evidence, we suspect that the fetal adrenal cortex is the primary modulator of these effects. However, further studies need to be conducted to support this hypothesis. Moreover, research is required to determine whether maternal adrenalectomy has long-term effects on offspring's neuroendocrine system that controls reaction to stress, digestion, immune function, mood and emotion, sexuality, energy storage, and/or expenditure.

The endocrine effects of fetoectomy have only been partially elucidated. Studies in baboons and rhesus monkeys have shown that fetoectomy—a process where the fetus is removed and the placenta is kept in situ—alters the maternal hypothalamic-pituitary-adrenal axis [41–43], eliminates the 24-hour steroid rhythm [44], and causes a loss in uterine activity [44]. Moreover, removal of the fetal adrenal gland during late gestation has been shown to cause a significant reduction in maternal plasma ACTH levels and a concomitant decrease in circulating cortisol and DHEA-S levels [41]. Interestingly, the drop in plasma DHEA-S concentrations between intact and fetoectomized animals was roughly 35% [41], which is equivalent to the difference between pregnant and nonpregnant animals [45]. Under normal physiological conditions, a drop in DHEA-S production is directly correlated with a decline in maternal estradiol and progesterone concentrations. This too was observed in fetoectomized baboons [42, 46] and monkeys [41, 43, 44]. Reintroducing DHEA-S to the placenta after fetoectomy was shown to restore placental estradiol production [46]. This data indicates that fetoectomy does not modulate placental function but rather that it affects the production of DHEA substrates needed by the placenta to generate endocrine hormones. Thus, estrogen production in the maternal circulation is an indicator of fetal adrenal steroidogenic activity. Taken together, this data strongly supports the idea that fetal adrenal cortex plays a primary role in modulating the feto-maternal endocrine communication.

7. Estrogen-Mediated Effects on Fetal Adrenal Function

The discovery that estrogen receptor alpha (ER-α) and beta (ER-β) are expressed in all zones of the fetal adrenal cortex suggests estrogen has a direct effect on fetal adrenal function. By binding to ER-α and ER-β, estrogen can induce both direct and indirect effects on the fetal adrenal cortex, and depending on plasma estrogen levels, it can stimulate or inhibit its cellular responsivity to ACTH [36]. As depicted in Figure 3, when high quantities of plasma estrogen are reached, estrogen acts directly on the fetal adrenal cortex

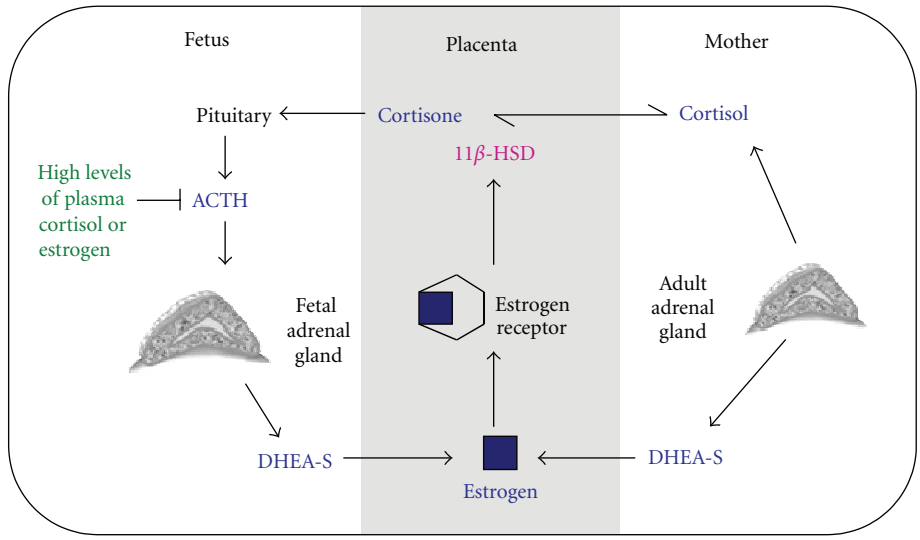

Figure 3: Representation of how placental estrogen during gestation modulates its own production. Placental estrogen has a positive and a negative feedback mechanism to ensure that physiological levels of estrogen are established. Estrogen produced by the placenta induces a positive feedback mechanism by promoting the conversion of cortisol to its biologically inactive metabolite cortisone through upregulation of 11β-HSD expression. Serum cortisone levels stimulate the fetal pituitary gland to produce ACTH which upregulates DHEA-S production from the fetal adrenal cortical zone and leads to placental estrogen production. In contrast, if placental estrogen concentrations are too high, placental estrogen can inhibit its own production by suppressing the responsiveness of the fetal adrenal zone to ACTH and lowering its production of DHEA-S. This figure has been modified from Albrecht et al. [36].

to suppress its responsivity to ACTH activity. This inhibits DHEA-S production from the fetal zone of the adrenal cortex and thereby lowers placental estrogen synthesis [36, 47, 48].

Comparative real-time PCR studies have shown that ER-α, ER-β mRNA, and protein expression is increased from mid- to late gestation in the definitive and transitional zones of the fetal adrenal gland, but not in the fetal zone [49, 50]. By lowering the number of available ERs on the fetal zone of the adrenal gland, the negative feedback system that suppresses DHEA-S production is desensitized, the estrogen, mediated positive feedback system is activated, and production of DHEA-S is upregulated (Figure 3).

Long-term cultures of human fetal adrenal cells have shown that estrogen binding to ER-α and ER-β can stimulate DHEA-S production through an indirect pathway that suppresses cortisol synthesis [51]. Cortisol is a stress hormone that maintains intrauterine homeostasis and influences the development and maturation of many fetal tissues including the lungs, liver, intestine, and the central nervous system [52]. Too much cortisol during pregnancy may have an adverse effect on fetal development by increasing maternal stress and blood pressure [52]. To protect the fetus from too much cortisol, the placental enzymes, 11β-hydroxysteroid dehydrogenase (11β-HSD), convert cortisol to the biologically inactive steroid, cortisone [4, 36, 53, 54]. This placental enzyme is regulated by estrogen and progesterone [55]. An increase in either estrogen or progesterone stimulates 11β-HSD to increase transplacental oxidation of cortisol to cortisone [55]. Low cortisol levels stimulate secretion of ACTH by the fetal pituitary gland, which in turn stimulates DHEA-S production from the fetal cortical zone, providing more substrate for placental estrogen production. In cases where ACTH levels become too high, the estrogen feedback loop may be turned off by stimulating cortisol synthesis (Figure 3) [36].

In late gestation, ACTH receptor mRNA expression is higher in the definitive and transitional zones than in the fetal zone of the human fetal adrenal cells. This suggests that ACTH promotes cortisol production during late gestation. Cortisol can inhibit estrogen synthesis by suppressing ACTH activity from the fetal pituitary gland. However, in late gestation, this pathway is likely not activated because high amounts of estrogen are required for initiation of parturition. Instead, high plasma cortisol levels in late gestation are likely converted to cortisone through the estrogen-mediated positive feedback system (Figure 3).

The available data has provided much mechanistic insight regarding the role of estrogen in modulating the development and function of fetal adrenal cortex [4, 36]. However, to more fully understand the role of estrogen in the fetal adrenal gland, it is important to better characterize the expression of coactivators and corepressors as well as the pattern of ER heterodimerization in different regions of the fetal adrenal gland. Moreover, three estrogen isoforms have been identified but the precise role of these isoforms in the physiology of pregnancy has not been elucidated. This may be one of the limiting factors in our current understanding of estrogen and fetal adrenal development. It is possible that structural differences between the three estrogen isoforms serve to induce selective effects at the level of nuclear receptor. Future research in this area will need to decipher the role of estradiol, estrone, and estriol on adrenal gland development and function.

8. Estrogen-Mediated Effects on Fetal Adrenal Growth and Development

Only one study has examined the effects of plasma estrogen levels on developmental differentiation of fetal cortical zones [11]. In this paper, fetal adrenal glands were obtained from baboons that had normal plasma estrogen levels and from those that had abnormally low estrogen levels due to administration of a highly specific aromatase inhibitor. Findings from this study revealed that fetal adrenal weight and volume increased 3-fold between mid- and late gestation and an additional 70% by aromatase administration, which decreased serum estradiol levels by 95%. The 70% rise in fetal adrenal growth among estrogen-deprived baboons was explained by a significant increase in the fetal zone. It was the only adrenal cortical site that exhibited any change in weight. This is an interesting finding because although ER-α and ER-β are expressed in all zones of the fetal adrenal cortex [36], loss of estrogen had a selective effect on the fetal zone, the primary site of stereocorticoid production. The observed increase in fetal zone size among estrogen-deprived baboons was associated with a 3-fold rise in fetal serum DHEA-S levels. Based on this evidence, researchers from this study concluded that plasma estrogen concentrations selectively repress both the morphological and functional development of the fetal zone in utero between mid- and late gestation [11]. According to these findings, it is possible that estrogen produced by the placenta feeds back to the fetal zone of the fetal adrenal cortex to restrain its growth and development. By controlling the size of the fetal zone, estrogen may be able to regulate the amount of DHEA-S being produced and, in turn, its own production. This is important because having high levels of estrogen can lead to endocrine disruption that may have deleterious effects on fetal growth and maturation [56].

9. Does Exposure to Environmental Estrogens Affect the Fetal Adrenal Gland Development and Function?

Exposure to environmental estrogens during perinatal life may have permanent long-lasting consequences on the overall growth and development of the fetus [56]. The fetus has a faster metabolic rate, a weaker immune system, and a lower concentration of detoxifying enzymes and liver metabolites compared to an adult, making it more sensitive to environmental disruptions [57]. Moreover, due to its small body size, exposure to environmental estrogens may cause chemical toxicity in the fetus at a concentration that has no adverse effect in an adult [57]. A growing number of commercially available products containing low doses of estrogen also increase the risk of estrogen accumulation in the mother as well as the fetus. Fetal cells are highly susceptible to change, so exposure to environmental estrogens, powerful chemical messengers, may induce adverse effects on fetal growth, development, and future health.

Although there are many environmental estrogens in the world today, their effects on fetal programming as well as fetal adrenal gland development and function have not been extensively studied. One exception is DES, a synthetic estrogen that used to be administered to pregnant women for prevention of miscarriages from the mid-1940s to 1970s [58–60]. Its use was discontinued, after several million offspring were exposed, because it was discovered that in utero exposure to DES causes major developmental changes in the offspring. It disrupts reproductive tract development and function at sexual maturity in 30% of the exposed population [61, 62]. Female offspring exhibit reduced fertility and significant increase in vaginal carcinomas, while male offspring suffer from reduced testicular size and sperm count [57]. Both genders, when exposed to DES during prenatal development, are susceptible to benign tumors in reproductive organs and autoimmune disorders [57]. Therefore, developmental changes induced by in utero exposure to DES have long-term programming effects on reproductive function. As previously mentioned, the primordial cells that give rise to reproductive organs are the same cells that give rise to the fetal zone of the fetal adrenal gland [4] (see Section 4), suggesting that prenatal exposure to DES may also disrupt adrenal gland development and function. Female rats treated with DES from gestation day 8 to 18 had a 30% enlargement in adrenal gland weight and a significantly lower concentration of circulating adrenal steroid hormones compared to the control group, including a 60% and 32% reduction in serum progesterone and estrogen levels, respectively [63]. The exposure to DES was also shown to reduce uterine contractions, prevent placental detachment from the uterine wall, and increase fetal death.

In examining fetal survival, it appears that the rat fetus is the most sensitive to DES treatment when exposure occurs between 18 and 20 days of gestation [63], suggesting that exposure to environmental estrogens has the most profound effect on fetal development in late gestation. This is not surprising because DES is structurally similar to estradiol and, as such, can bind to ERs to induce estrogen-like effects [64, 65]. Exposure to exogenous estrogens during late gestation has the potential to activate the negative feedback loop to inhibit DHEA-S production from the fetal adrenal cortex because the fetus is already exposed to high levels of endogenous estrogen. Moreover, such high levels of estrogen could cause a decrease in blood gonadotropins (i.e., FSH, LH) which in turn could slow down follicular development and depress follicular synthesis of progesterone and testosterone [66–68]. The aromatase enzyme converts testosterone to estradiol, and thus, this pathway may also be turned off [69]. Depressed fetal zone function and decreased follicular synthesis of testosterone may in combination suppress de novo synthesis of estrogen. As shown by Zimmerman et al. [63] dams treated with DES had a 60% and 32% reduction in progesterone and estrogen levels, respectively, compared to the control group.

DES is no longer administered clinically for prevention of miscarriages but remains a useful compound for investigating the role of estrogen on fetal growth and development [70, 71]. Findings from DES studies are currently being used to better characterize the biological effects of other estrogenic compounds, including dietary estrogens [70–72] and BPA. The most commonly studied dietary estrogens during development are soy isoflavones because of their abundance

in soy-based foods such as soy milk, tofu, miso, and soy protein [73]. Traditional Asian diets are rich in soy-based foods, and thus, many Asian populations consume between 15 and 40 mg of isoflavones a day [74–77]. At this level of exposure, there is no evidence that soy-based foods or isoflavones have adverse effects on human health. However, there are substantial differences in the form (fermented versus nonfermented), dose, and timing of isoflavone exposure between the traditional Asian and current Western diets [78]. Soybeans used for production of soy-based foods undergo some type of processing. Soybeans used in traditional Asian diets undergo germination, cooking, roasting, and fermentation while those used in Western diets employ more modern methods such as fractionation and extraction. The differences in soybean processing can affect the bioavailability of isoflavones and digestibility of soy protein through removal of indigestible sugars and inactivation of enzymes [79]. Studies show that fermented soy foods (i.e., miso and tempeh) are rich in aglycones that are more bioactive than the glycone form of isoflavones [79]. However, many unfermented soy foods available on the Western market can contain a substantially higher amount of total isoflavones (aglycone and glycone forms of isoflavone). For example, one soy burger contains 15–25 mg of isoflavones. A soy protein bar, often marketed as a meal replacement bar and sometimes used in weight management strategies, can contain 60 mg of isoflavones per serving. Consuming three to four servings of these soy foods a day in the Western World can exceed the level of isoflavone exposure reported in Asian populations (100–200 mg/day versus 15–40 mg/day). It is also possible that North Americans respond differently to soy foods than Asian populations because of their difference in timing of isoflavone exposure. Asian populations consume soy foods throughout their entire life, except for the brief neonatal period when most infants are breastfed [80, 81]. In contrast, in North America, soy is primarily consumed by infants fed soy protein formula or by health conscious adults eating a plant-based diet for general health and/or lowering risk of developing a chronic disease such as heart disease. Pregnant women consuming soy foods expose their fetus to isoflavones. Studies show that isoflavones are transferred readily across the placenta in humans and rodent models [82, 83]. Cord serum concentrations of genistein, daidzein, and equol correlate closely with maternal serum concentrations and isoflavones remain longer in fetal than maternal circulation [84]. Thus, depending on maternal diet, there is potential for a fetus to be exposed to substantial levels of isoflavones in utero.

The spatial and structural arrangement of isoflavones is slightly different from that of estrogen, and thus, they can induce both estrogenic and nonestrogenic effects. Findings from two studies suggest that soy isoflavones may be able to alter fetal adrenocortical function [85, 86]. Feeding daidzein to late pregnant sows downregulates ER-β expression in the hypothalamus, which likely affects the regulation of fetal zone DHEA-S production [85]. In cultured human fetal adrenocortical cells, soy isoflavones (i.e., genistein and daidzein) decrease ACTH-stimulated cortisol production by inhibiting the expression of 21-hydroxylase (P450c21) but had no effect on DHEA-S production [86]. Further research is required to elucidate whether in utero exposure to soy isoflavone can alter DHEA-S production in vivo and whether there are other effects that may be subtle but nonetheless have lifelong implications to health.

10. The Effects of Environmental Estrogen on Fetal Adrenal Cortex May Be Mediated through Epigenetic Changes

Although not fully understood, it has been hypothesized that estrogenic compounds during perinatal life can modulate epigenetic gene regulation, resulting in permanent effects that alter responses in later life and transgenerational inheritance. The most commonly studied epigenetic modification is DNA methylation, used by mammals to regulate gene transcription, chromosomal positioning, activation of X-chromosome, and gene imprinting. From a molecular perspective, DNA methylation is a term used to denote the attachment or substitution of a methyl group for a hydrogen atom on a cytosine residue. In vertebrates, DNA methylation depends on an enzyme known as methyltransferase which recognizes the CpG dinucleotides of various palindromic sequences and then catalyzes the transfer of the methyl group on the cytosine residue. As a result, modulators of DNA methylation are typically methyl donors or acceptors. However, estrogen-based compounds, which have been postulated to play a role in DNA methylation, cannot donate or accept a methyl group. Instead, it is believed that estrogenic compounds are able to enhance histone acetylation through interaction with ERs and that this interaction may open up the methylation region [87]. When this happens, methyl donors and cofactors including folate, choline, and SAM may stimulate DNA methylation [88].

Fetuses are exposed to high concentrations of folate during early stages of development because mothers are advised to consume folate supplements during pregnancy to lower the incidence of spina bifida [89–91]. As a result, the combination of folic acid with high concentrations of circulating estrogens during early stages of development may cause an additive or synergistic effect on DNA methylation [88]. These changes in DNA methylation patterns have the potential to induce both beneficial and adverse effects on the developing genome. Future research is required to confirm whether early exposure to environmental estrogens can alter the plasma estrogen profile enough to induce long-term programming effect on the developing fetus.

To date, only one study has investigated whether soy isoflavones can alter DNA methylation patterns [88]. This study found that exposure to genistein resulted in hypermethylation of six CpG sites in the agouti mouse genome, which decreased the incidence of adult-onset obesity. ER-α, which has been shown to play a role in adrenal gland development and function, has at least eight promoters that contain CpG islands and are thus tightly regulated by DNA methylation [92]. Thus, it is possible that by regulating DNA methylation isoflavones as well as other environmental estrogens may be able to modulate adrenal gland growth and development.

11. Conclusion

The fetal adrenal cortex, together with the placenta and the maternal adrenal cortex, forms a unique fetomaternal endocrine system that regulates estrogen production during development. To date, notable progress has been made in understanding the embryogenesis and morphology of the fetal adrenal gland, the role of steroidogenic enzymes and nuclear receptors in differentiation of cortical zone function, and the process by which estrogen regulates its own production in the fetal adrenal cortex. However, there is still much work to be done in characterizing the effects of in utero exposure to environmental estrogens on fetal adrenal gland development and function. The available evidence suggests that exposure to environmental estrogens such as DES and soy isoflavones can reduce cortisol and DHEA synthesis in the fetal adrenal cortex as well as decrease placental estrogen production. The decrease in placental estrogen production during sensitive stages of development has the potential to alter gene transcription and DNA methylation patters of cells, suggesting that environmental estrogens may be able to induce epigenetic changes in the developing fetus.

Future studies on this topic should examine whether exposure to environmental estrogens can provide a causal mechanism to explain some of the endocrine-mediated adult-onset diseases. By integrating current knowledge of developmental biology with concepts of endocrine regulation, future studies will be better directed into uncovering the dis ruptions induced by environmental estrogens on fetal adre nal gland development and function.

Abbreviations

ACTH: Adrenocorticotropic hormone
CRH: Corticotrophin-releasing hormone
DHEA: Dehydroepiandrosterone
ER: Estrogen receptor
DHEA-S: Dehydroepiandrosterone-sulfate
P450c17: 17α-hydroxylase
HSD: Hydroxysteroid dehydrogenase.

Conflict of Interests

J. Kaludjerovic and W. E. Ward have no conflict of interests.

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Research Article

Mitochondrial Respiration Is Decreased in Rat Kidney Following Fetal Exposure to a MaternalLow-Protein Diet

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Maternal protein restriction in rat pregnancy is associated with impaired renal development and age-related loss of renal function in the resulting offspring. Pregnant rats were fed either control or low-protein (LP) diets, and kidneys from their male offspring were collected at 4, 13, or 16 weeks of age. Mitochondrial state 3 and state 4 respiratory rates were decreased by a third in the LP exposed adults. The reduction in mitochondrial function was not explained by complex IV deficiency or altered expression of the complex I subunits that are typically associated with mitochondrial dysfunction. Similarly, there was no evidence that LP-exposure resulted in greater oxidative damage to the kidney, differential expression of ATP synthetase β-subunit, and ATP-ADP translocase 1. mRNA expression of uncoupling protein 2 was increased in adult rats exposed to LP *in utero*, but there was no evidence of differential expression at the protein level. Exposure to maternal undernutrition is associated with a decrease in mitochondrial respiration in kidneys of adult rats. In the absence of gross disturbances in respiratory chain protein expression, programming of coupling efficiency may explain the long-term impact of the maternal diet.

1. Introduction

Retrospective associations between low weight or thinness at birth and risk of cardiovascular disease and type 2 diabetes gave rise to the hypothesis that maternal nutritional status may be one of a number of factors that programme long-term risk of disease [1–4]. This hypothesis has received strong support from studies of small and large animal species, which overwhelmingly indicate that exposure to undernutrition in pregnancy, whether specific to macronutrients [5] or micronutrients (e.g., iron) [6], or in the form of lower overall food intake [7], programmes risk of adult hypertension, glucose intolerance, insulin resistance, and dyslipidaemia [5–9]. Maternal protein restriction during rat pregnancy has been widely reported to impact upon blood pressure in the exposed offspring and the hypertension observed in such animals has partly been linked to programming of renal development [10].

Maternal protein restriction brings about early-life programming through remodelling of specific tissues [11]. In the kidney, the number of nephrons is reduced through exposure to maternal undernutrition, and with ageing, low-protein-exposed animals develop glomerular injury and progressive loss of renal function [10, 12, 13]. There is evidence to suggest that this is, at least in part, mediated by decreased activity of antioxidant enzymes and an associated increase in oxidative injury [13, 14]. The basis for these effects of maternal diet is unclear and whilst programming is widely believed to be driven by epigenetic mechanisms [15], there is no specific evidence that there is differential regulation of genes which determine antioxidant-reactive oxygen species balance. One hypothesis is that the programming is achieved through altered regulation of coupling efficiency.

Mitochondrial respiration is the major source of reactive oxygen species in mammalian tissues, with superoxide

radicals emanating from the respiratory chain at several points, most notably the transfer of electrons by complexes I and III. Mitochondrial dysfunction has been linked to many of the diseases also associated with early-life programming, including atherosclerosis, hypertension, renal dysfunction, and diabetes [16]. There are some reports that mitochondrial dysfunction may play a role in the declining physiological function in animals exposed to less than optimal maternal nutrition during fetal life. Most studies have considered the impact of maternal overnutrition. Offspring of mice rendered obese prior to pregnancy exhibit functional deficits in mitochondrial function at the early embryonic stage and postnatal [17]. Such changes are associated with an increase in mitochondrial DNA copy number and reactive oxygen species generation. In rats exposed to a maternal high-fat diet, kidney mitochondrial DNA was decreased, and expression of the mitochondrial genome in aorta was downregulated [18]. Mortensen and colleagues reported that mice exposed to maternal protein restriction showed changes in expression of mitochondrial genes, with differential responses to diet in liver and skeletal muscle [19]. However, no functional phenotype was described in this study. Together such findings suggest that mitochondria may be targets for programming in response to a number of insults, but to date no study has integrated a study of programmed changes in gene or protein expression with measures of mitochondrial function in the kidney. The aim of this study was therefore to explore the hypothesis that the renal dysfunction that is associated with maternal protein restriction is a consequence of programming of mitochondrial function via alterations in the expression of mitochondrial genes and proteins and to the regulation of coupling efficiency.

2. Materials and Methods

2.1. Animals. Two separate animal experiments were carried out under license from the UK Home Office, in accordance with the Animals (Scientific Procedures) Act 1986. In both trials, virgin female Wistar rats (Harlan, UK) were mated at between 180 and 225 g weight, with stud males. Upon confirmation of pregnancy by the presence of a semen plug, the female rats were randomly allocated to be fed one of two isocaloric synthetic diets (18% casein, CON; 9% casein, LP), as reported previously [20, 21]. In the first experiment 10 pregnant rats were fed the semi-synthetic diets (5 CON; 5 LP) until they delivered pups at 22d gestation. Upon delivery of pups, the mothers were transferred to a standard laboratory chow diet (B&K Universal Ltd, Hull, UK, rat and mouse diet) and the litters were culled to a maximum of 8 pups, (4 males and 4 females). This minimized variation in nutrition during the suckling period. The offspring from the two groups therefore differed only in terms of their prenatal dietary experience. At 16 weeks after birth, 2 male animals from each litter were culled using carbon dioxide asphyxia and cervical dislocation. For one animal in each litter, the right kidney was collected, snap-frozen in liquid nitrogen, and stored at -80°C for later analysis of gene expression. Both kidneys were collected from a second male from each litter, and these were used for fresh preparation of mitochondria and analysis of respiratory chain function. In the second experiment, 12 pregnant rats were fed the semisynthetic diets (6 CON; 6 LP) until they delivered pups at 22d gestation. The protocol for maternal feeding and for weaning the offspring was as described above. At 4 weeks after birth, the pups were weaned onto chow diet and half of the animals were then culled using carbon dioxide asphyxia and cervical dislocation. The right kidney was collected, snap-frozen in liquid nitrogen, and stored at -80°C. The remaining animals were maintained on standard laboratory chow and culled at 13 weeks of age. Kidneys were collected, snap-frozen in liquid nitrogen, and stored at -80°C.

Kidney samples from male offspring used for histochemistry, immunohistochemistry, and analysis of mitochondrial DNA copy number (experiment two) were resnap frozen in isopentane and stored in a -80°C freezer. Serial sections were obtained using a cryostat at -20°C to a thickness of 15 μm and then stored at -80°C after air drying. All sections were air-dried for 90 minutes at room temperature prior to use.

2.2. Histochemistry

2.2.1. Complex IV Histochemistry. Mitochondrial respiratory chain activity was determined using sequential complex IV(COX)/complex II (succinate dehydrogenase [SDH]) histochemistry. Complex IV activity was detected using COX medium. Any cells with complex IV activity stained brown. To assess whether there was evidence of complex IV deficiency, sections were washed twice in phosphate-buffered saline and the SDH medium, an indicator of complex II activity, was applied, blue staining any cells that failed to stain brown (indicating complex IV activity). Sections were dehydrated in 70%, 95%, and 100% ethanol and mounted in DPX resin. Images were captured using a Zeiss microscope and analysed using Adobe Photoshop CS4. Areas of mitochondria were highlighted, and intensity within each area was calculated using the measurement tool.

2.3. Immunohistochemistry. Immunohistochemistry was used to determine variation in expression of a variety of proteins including some oxidative phosphorylation (OXPHOS) proteins and some markers for oxidative stress. Complex I-19 (CI-19; antibody diluted 1 in 300), Complex I-20 (CI-20; 1 in 50), and complex IV subunit I (1 in 200) are subunits of the OXPHOS complexes that are commonly affected in mitochondrial dysfunction. They are encoded by the mitochondrial genome. Complex II-70 (CII-70; 1 in 300) is rarely affected in mitochondrial dysfunction. It is encoded by the nuclear genome. Phospho-histone H_2AX (H2AX; 1 in 200) and 8-Hydroxy-2′-deoxyguanosine (8-OHdG; 1 in 200) are markers of oxidative damage to DNA, and superoxide dismutase 2 (SOD2; 1 in 1000) is an antioxidant produced in the mitochondria in response to reactive oxygen species.

All sections for immunohistochemistry were fixed in 4% paraformaldehyde solution for 10 minutes, followed by alcohol permeabilisation. Nonspecific binding was blocked using an avidin biotin blocking kit (Sigma, UK), followed

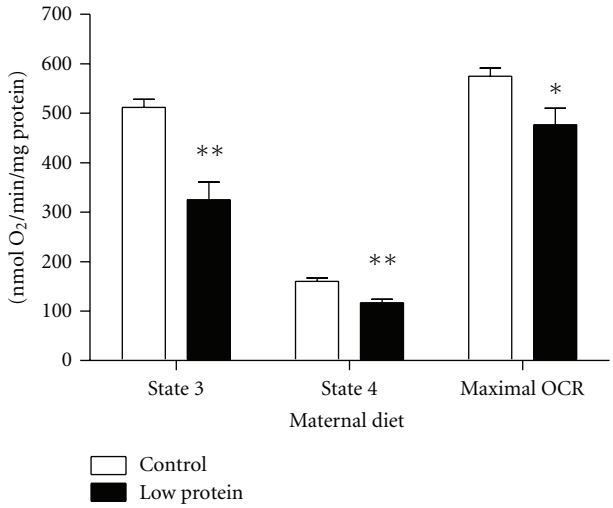

Figure 1: Respiration rates in mitochondria from rat kidney. Respiration rates in state 3, state 4 and maximal respiration rates were determined in isolated mitochondria using an oxygen electrode. Duplicate measurements were performed on each preparation and averaged. Values are means ± S.E.M. from 5 independent preparations for each group. $^{*,**}P < 0.05, P < 0.01$ compared with controls in the same condition.

by 1% normal goat serum (Sigma). The sections were incubated with primary antibodies for 90 minutes at room temperature. The secondary antibodies (either antimouse rat adsorbed or antirabbit biotinylated) were incubated for 30 minutes, followed by ABC (Sigma) for a further 30 minutes and DAB (Sigma) for approximately 6 minutes. Sections were then dehydrated and mounted, as for COX/SDH histochemistry. Images were captured using a Zeiss microscope and analysed using Adobe Photoshop CS4.

2.4. Mitochondrial DNA Copy Number. For laser microdissection, cryostat sections (15 μm thick) were mounted onto membrane slides (Leica), and SDH histochemistry was performed. To determine mtDNA copy number, tissue (approximately 1000 μm^2) was dissected from proximal convoluted tubules using a Leica laser micro-dissection microscope (Leica LMD). DNA extraction was carried out using a lysis buffer containing tris-HCl, tween 20, and proteinase K.

For estimation of mtDNA copy number, the control region (D-loop) of rat mtDNA was amplified using the primers and probe as follows: forward primer (5′-GGT TCT TAC TTC AGG GCC ATC A-3′), reverse primer (5′-GAT TAG ACC CGTTAC CAT CGA GAT-3′), and probe (6FAM-TTG GTT CAT CGT CCA TAC GTT CCC CTT A-TAMRA). The PCR program consisted of a 2 min incubation at 50°C, 10 min at 95°C, and 40 cycles of amplification; 15 sec denaturation at 95°C; 1 min at 60°C for hybridization of probes, primers and DNA synthesis. Mitochondrial DNA copy number was calculated per μm^2 from a standard curve.

2.5. Rat Kidney Mitochondrial Function. Whole kidneys from experiment two were chopped and minced, then homogenised in STE (250 mM sucrose, 5 mM Tris/HCl at pH 7.4, and 2 mM EGTA) medium using a Teflon dounce homogenised. Mitochondria were prepared by differential centrifugation at 4°C using STE, adapting a method de-scribed by Rolfe et al. [22]. The protein concentrations of the mitochondria preparations were determined by the Biuret method with bovine serum albumin (BSA) as a standard [23].

2.5.1. Mitochondrial Respiration. The assay medium consisted of 120 mM KCl, 5 mM KH_2PO_4, 3 mM HEPES, 1 mM EGTA, and 0.3% (w/v) BSA at pH 7.2. Respiration was measured inside a sealed Clark oxygen electrode (Rank Brothers Ltd, UK) at a temperature of 37°C. The medium was air-saturated and assumed to contain 406 nmol of oxygen/mL [24]. Electrodes were monitored for linearity from 100% to 0% air saturation for each mitochondrial preparation. Mitochondria were resuspended to 0.35 mg of protein/mL in assay medium containing rotenone (5 μM) and succinate (4 mM). Excess ADP (1 mM) was added to establish state 3 respiration [25]. Maximum state 3 respirations were established by adding carbonyl cyanide p-trifluoromethoxyphenyl-hydrazone, up to 5 μM.

2.5.2. Measurement of Proton Conductance. Triphenylmethylphosphonium (TPMP), the potential sensitive probe was used to measure membrane potential by detecting the external TPMP in the medium. This was done in the presence of rotenone (5 μM), nigericin (80 ng/mL to clamp pH gradient to 0), and oligomycin (at 1 μg/mL to inhibit ATP synthesis). The electrode was calibrated with sequential 1 μM additions up to 5 μM TPMP. Succinate (4 mM) was used to start respiration. Membrane potential and oxygen consumption were progressively inhibited through successive steady states using up to 4 mM Malonate [22]. This was continuously monitored and data displayed on the computerised system that used Chart 5 software (AD instruments, UK). At the end of each experimental run, 0.5 μM FCCP was added to dissipate the membrane potential. This released all internal TPMP into the medium allowing correction for any small electrode drift. TPMP binding correction was assumed to be 0.4 μl/mg protein for kidney [26]. The respiration at each steady state was plotted against the corresponding membrane potential.

2.6. Quantitative Real-Time PCR. Quantitative real-time PCR was used to determine mRNA expression, using a Roche LightCycler [27]. Total RNA was extracted from the kidneys (experiment 1) using a TRIzol extraction (Invitrogen, UK). The RNA was DNase-treated (Promega, UK) to remove any genomic DNA and then extracted using a phenol-chloroform extraction followed by ethanol precipitation. Copy DNA (from 0.5 μg RNA) was reversely transcribed using Maloney murine leukemia virus RT (Promega). Template-specific primers were designed for UCP2: (forward primer: GACCTCATCAAAGATACTCCTGAAb and reverse primer: CAATGACGGTGGTGCAGAAG); ATP Synthetase β-subunit (forward primer: CCACCAAGAAGGGCTCGAT and reverse primer: GGCAGGGTCAGTTCAGGTCAT); ATP-ADP

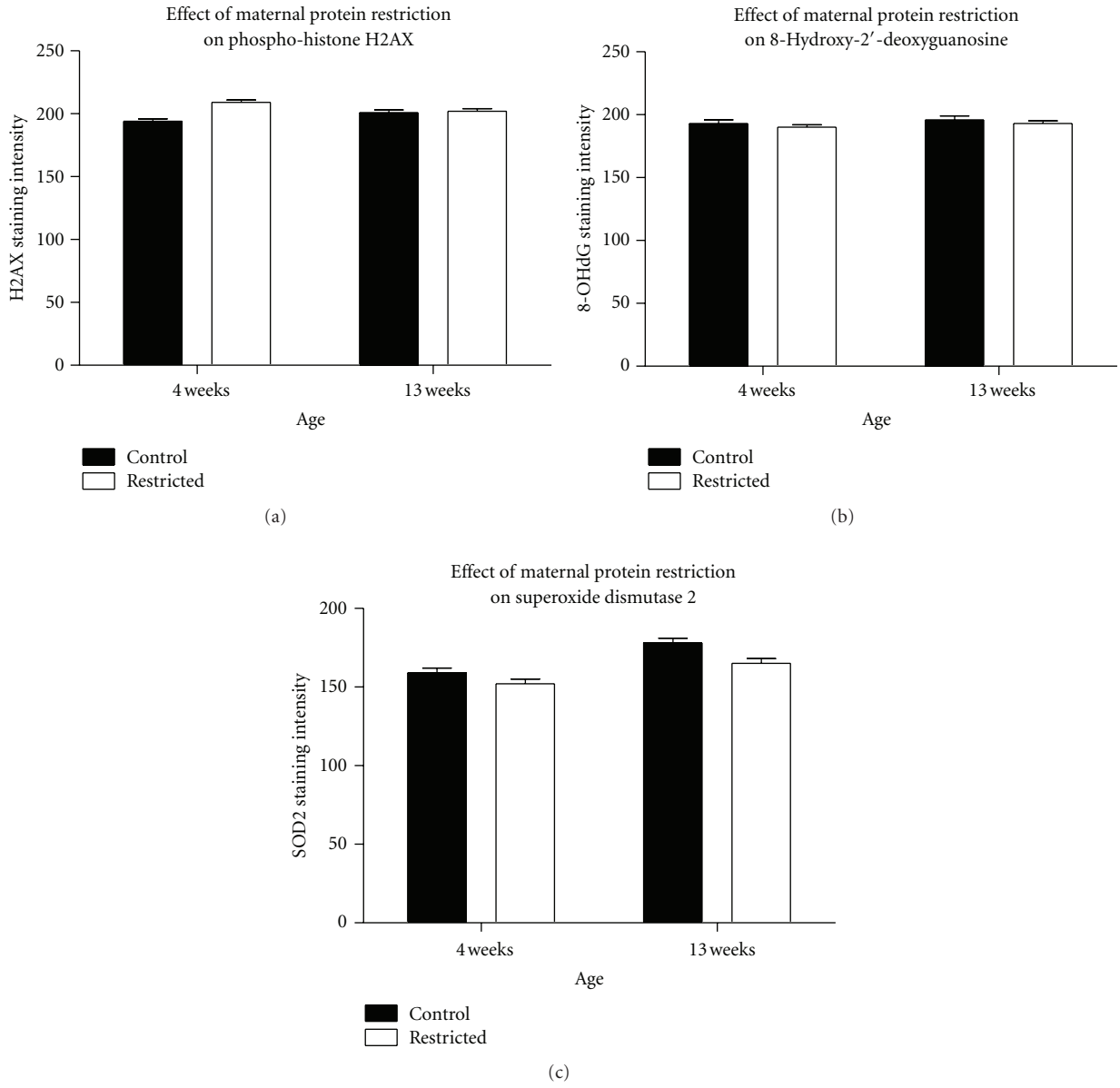

Figure 2: Oxidative stress in kidney tissue as measured by immunohistochemistry. Markers of oxidative stress within the mitochondria were unaffected by maternal protein restriction. Values are means ± S.E.M. $n = 4$–6.

translocase 1 (forward primer: CCCGATCGAGAGGGTCAAA and reverse primer: TGTACTGTTTCTCTGCACTGATCTGT); the housekeeping gene β-actin using Primer Express Version 1.5 (Applied Biosystems, USA). A standard curve was produced using a pool of the copy DNA, and all samples were normalised to β-actin expression. β -actin expression was not influenced by maternal diet.

2.7. Western Blotting. Expression of UCP2 (primary antibody; Calbiochem, cat. number 662047) and ATP synthetase β-subunit (primary antibody; Sigma cat. number A9728) protein was determined in mitochondrial preparations by Western blotting using previously described methods [28]. Expression was normalised to β-actin.

2.8. Statistics. Student's t-test was used to determine statistical significance between maternal dietary groups. All data are shown as mean ± SEM. $P < 0.05$ was accepted as statistically significant.

3. Results and Discussion

The basis for this study was the observation that ageing rats exposed to maternal protein restriction *in utero* exhibit glomerular injury and loss of renal function, both of which are possibly mediated by an imbalance of reactive oxygen species and antioxidant protection [12, 13]. Mitochondria are the primary source of reactive oxygen species, so functional studies of mitochondria isolated from kidneys of

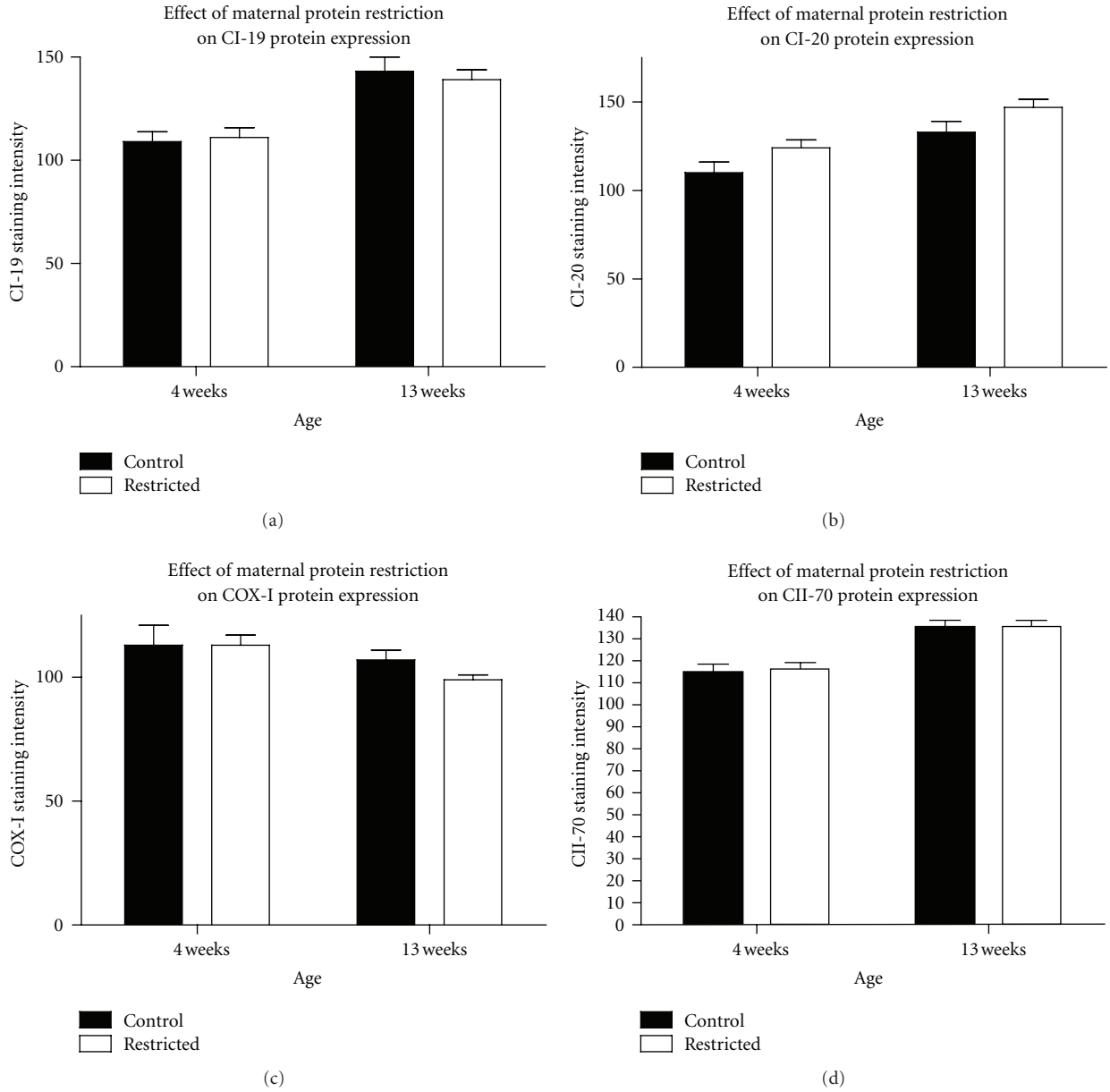

Figure 3: OXPHOS pathway proteins in kidney tissue as measured by immunohistochemistry. Protein expression of key subunits from complexes I and IV were unaffected by maternal protein restriction. Values are means ± S.E.M. $n = 4$–6.

control and protein-restricted offspring, were performed. As shown in Figure 1, mitochondrial respiration in kidneys from adult rats was reduced by maternal protein restriction. State 3 and state 4 respiration rates were approximately one-third lower in offspring of LP dams compared to the offspring of control fed dams ($P < 0.01$). The maximal respiratory rate for the renal mitochondria was reduced by 17% in the LP group. State 3 represents the actively respiring state, whilst state 4 describes respiration in the absence of ATP synthesis. The data are consistent with a general downregulation of mitochondrial function. Decreased mitochondrial respiration is associated with a number of disease states, including metabolic disturbance associated with obesity. Essop and colleagues reported that in hearts of young, obese, prediabetic rats, state 4 respiration was reduced, and the capacity of isolated mitochondria to recover state 3 respiration following oxygen depletion was impaired [29]. It is known that rats exposed to maternal protein restriction in fetal life develop insulin resistance and glucose intolerance with ageing [9, 28], so the current observation of impaired mitochondrial respiration is of interest in this context. It was also noted that although maximal respiratory rate declined as did state 4, the state 3 respiratory rate declined further, leaving a greater reserve capacity and a lower respiratory control ratio. This could be of importance with regard to the programmed animals' ability to cope with challenges, possibly suggesting an inappropriate adaptation in an attempt to align supply and demand. Further studies

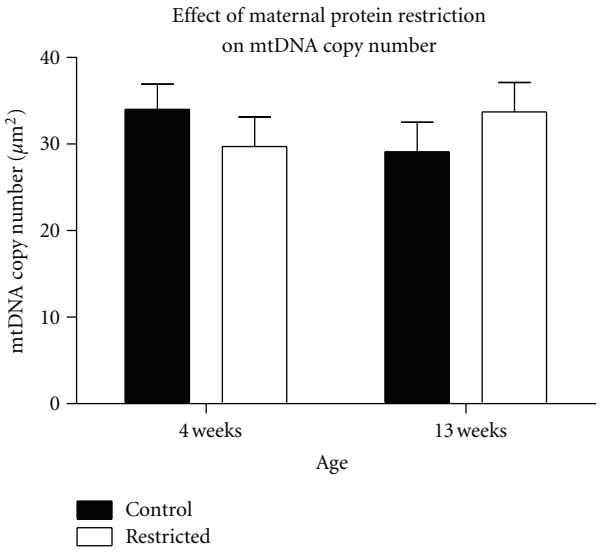

Figure 4: Mitochondrial DNA copy number in kidney as determined by PCR. mtDNA copy number was unaffected by maternal protein restriction. Values are means ± S.E.M. $n = 10$.

need to address the ability of LP exposed animals to cope with challenges to their regulation of bioenergetics.

No other studies have determined the effects of maternal nutrition upon mitochondrial respiration and its regulation by alterations in proton conductance. The reduced rate of respiration that we observed could appear to contradict the assertion that altered mitochondrial respiration results in greater release of reactive oxygen species, causing more oxidative injury to the tissues. This interpretation was further supported by the observation that maternal protein restriction did not impact upon any of the markers of oxidative stress that were measured (Figure 2). Protein expression of H2AX and SOD2 were unaffected by maternal protein restriction, and there was no evidence of increased 8-OHdG. The lack of evidence of oxidative processes may be indicative of mitochondrial reactive oxygen species production playing no role in renal injury associated with maternal protein restriction. Alternatively, it may be the case that oxidative injury occurs much later in life. Joles et al. found little evidence of oxidative damage in kidneys of rats exposed to prenatal protein restriction, aged under 18 months, although proteinuria and declining function occurred in much younger animals [12, 13]. It may be that what is being observed here is that these programmed animals successfully adapted their respiratory function to cope with their current environment, which when compared to control animals appears to be early mitochondrial dysfunction. It only develops into a potentially damaging process with ageing, when they are unable to cope with decreasing cell number. It is possible that the time points selected in this study were too early, but clearly a bioenergetics approach is of utility as it appears to reveal changes that occur prior to tissue damage and to offer some predictive power. The methods used in this study could be used to test interventions in programmed animals that have the potential to prevent or delay age-related disease. It would be highly desirable to establish noninvasive biomarkers of mitochondrial function, for example, suppressed serum coenzyme Q10 or elevated urinary concentrations of Krebs cycle intermediates, which would enable findings of such studies to more readily translate into human interventions.

The observed reduction in mitochondrial respiration could be suggestive of deficits in expression or activity of the electron transport chain. Mitochondrial control theory proposes that the level of electron transport chain activity must fall below critical thresholds before any deficits of mitochondrial membrane potential, oxygen consumption, or ATP synthesis can be observed [30]. Lower state 3 respiration of the low-protein exposed animals could possibly be associated with lower complex IV activity. However, COX/SDH histochemistry showed no indication of complex IV deficiency in any of the kidney tissues at 4 or 13 weeks of age in offspring of either control or protein restricted dams (data not shown). Sections stained consistently for complex IV, with no blue areas which would indicate a lack of COX activity. Similarly, immunohistochemistry indicated that maternal protein restriction had no effect on the protein expression of CI-19, COX I, or CII-70 in the kidney (Figure 3). Expression of CI-20 was slightly increased by maternal protein restriction ($P = 0.022$). However, when variability of CII-70 was used to adjust the data, this increase was no longer significant ($P = 0.097$). The data, therefore, indicate no gross impact of the maternal diet upon expression of the electron transport chain, but this does not exclude the possibility that activity of complexes I-III may be impaired. Although there was some variation in renal mitochondrial DNA copy number with age, maternal protein restriction had no effect on copy number at either 4 or 13 weeks of age (Figure 4). This suggests that differences in respiration rates were unrelated to variation in mitochondrial yields from the kidneys. Taylor and colleagues reported that maternal diet impacted upon mitochondrial copy number in rat kidneys [18]. This, however, was evidence of programming related to over- rather than under-nutrition, suggesting that the mechanistic basis of such programming may vary depending upon the nature of the dietary insult. Mitochondrial copy number and the expression of the mitochondrial genes decline with ageing [31]. In keeping with the observation that oxidative injury develops with age [13], it may be hypothesized that maternal protein restriction could impact upon the ageing process, bringing about an imbalance of oxidative and antioxidant processes. It is well established that exposure to maternal protein restriction shortens lifespan and increases markers of cellular ageing [13, 15, 32].

Lower state 3 respiration may also be associated with impaired ATP synthesis. To assess this as a potential target for nutritional programming, we determined the mRNA expression of ATP synthetase and ATP-ADP translocase 1, which is one of the proteins responsible for movement of ATP and ADP across the innermitochondrial membrane. ATP synthetase β-subunit and ATP-ADP translocase 1 mRNA expression were unaffected by maternal protein restriction (Figure 5). Uncoupling protein 2 allows protons to cross the

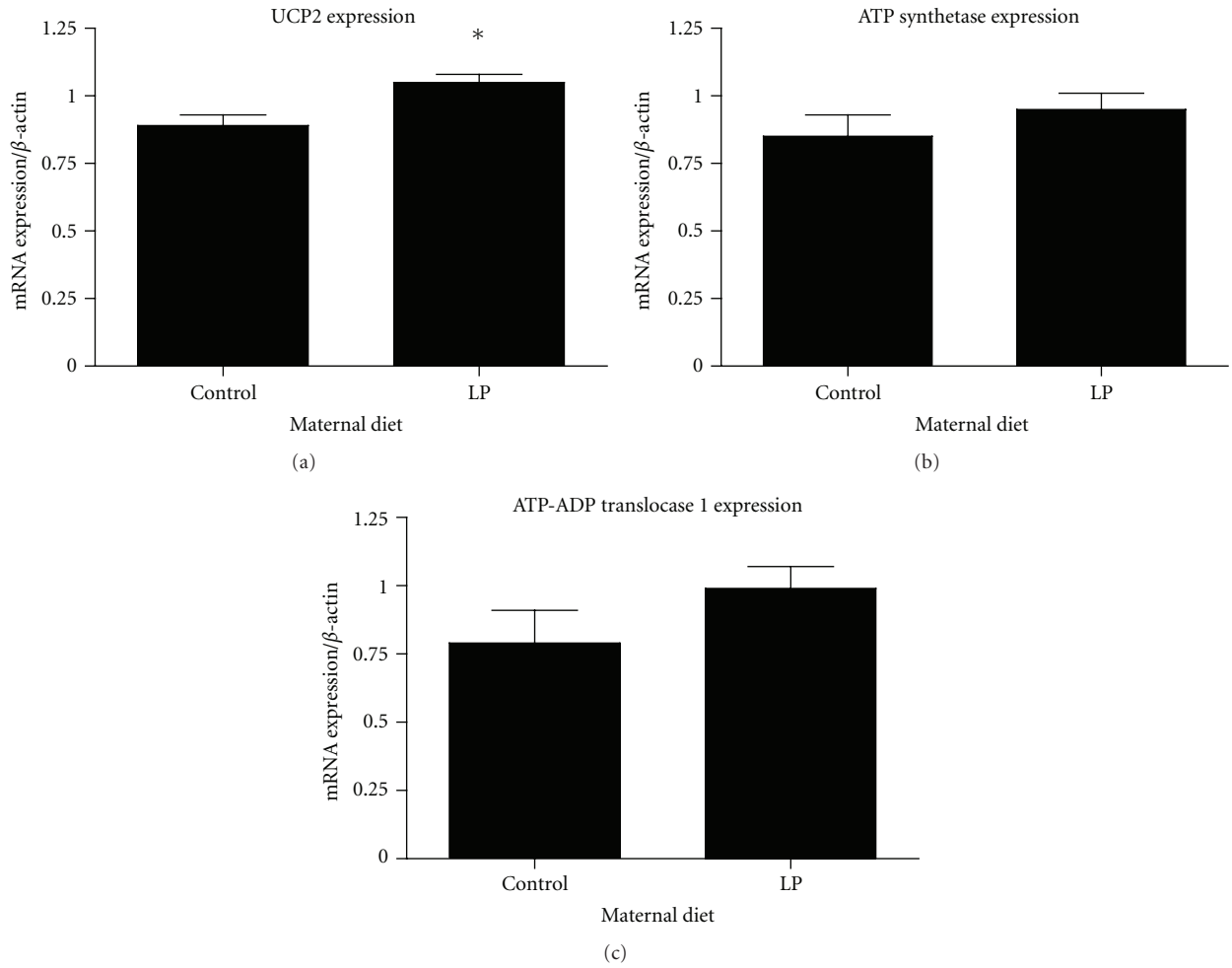

Figure 5: Gene expression of mitochondrial targets measured by RT-PCR. mRNA expression UCP2 was increased in offspring of protein-restricted dams. ATP Synthetase β-subunit and ATP-ADP translocase 1 were unaffected by maternal protein restriction. Values are means ± S.E.M. $n = 5$ animals per group.* $P < 0.05$ compared with control.

inner mitochondrial membrane without ATP synthesis and reduces the mitochondrial membrane potential. In some tissues, UCP2 reduces oxidative stress [33], which corresponds to the theory that mild uncoupling can protect against ROS production becoming excessive at high membrane potentials [34]. Reduction of mitochondrial respiration may be associated with inhibition of ATP synthesis. The lower state 4 respiration of the offspring of protein restricted dams is, however, suggestive of reduced uncoupling, particularly as there was no evidence of altered expression of ATP synthetase. We found that expression of UCP2 mRNA was significantly increased in offspring of protein restricted dams (Figure 5). The difference in the expression of UCP2 mRNA was not, however, reflected at the protein level (Figure 6). In the absence of clear and consistent evidence of UCP2 overexpression, further experiments are required to assess the possibility that maternal diet impacts upon mitochondrial respiration through this mechanism. Gnanalingham and colleagues have reported that, in sheep, maternal undernutrition increases the fetal expression of UCP2 [35]. This is consistent with the notion that the programming effect is to tighten coupling to improve efficiency and what is seen when looking at the mitochondria after living on a normal diet is the compensated state achieved by increased uncoupling. When we measured membrane potential and proton leak (Figure 7), it was evident that the LP animals were operating at a lower membrane potential (state 4) but consuming the same amount of oxygen at all common membrane potentials observed. This seems consistent with what would be expected if they had been programmed to have a tightened coupling efficiency (higher membrane potential with lower oxygen consumption) but then uncoupled in response to the plentiful food supply. This would presumably be at the cost of being able to further uncouple in challenging situations where the control animals would still be able to adapt. Further research needs to be done to explore how the programmed animals differ from control animals in response to specific challenges at this level of regulation.

The present study has identified a programmed deficit of mitochondrial function in the kidney following maternal undernutrition. Whilst the evidence indicates a potential role for UCP2 in mediating this effect, the work did not

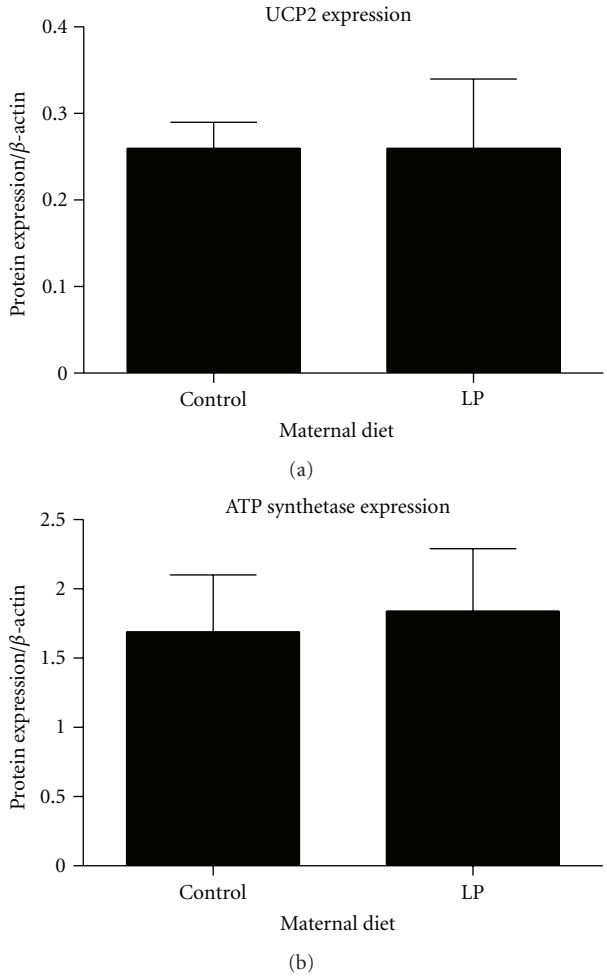

Figure 6: Expression of UCP2 and ATP synthetase β-subunit, determined by Western blot. Protein expression of UCP2 and ATP Synthetase β-subunit were unaltered by maternal protein restriction. Values are means ± S.E.M. $n = 5$ animals per group.

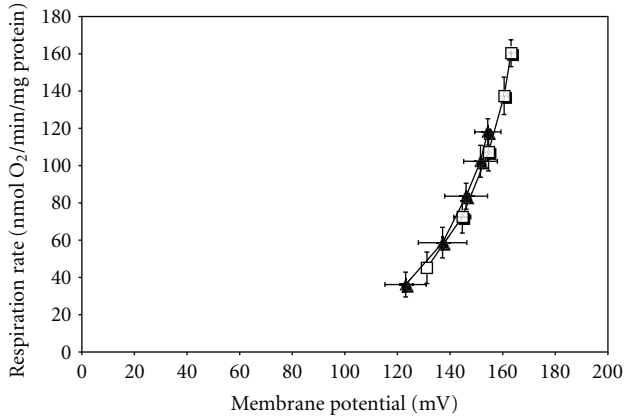

Figure 7: Proton leak of mitochondria from kidney showing controls and low protein fed rats. Empty box □: control; filled triangle ▲: low-protein. The highest common potential was 154 mV. Duplicate measurements were performed on each preparation and averaged. Values are means ± S.E.M. from 5 independent preparations for each group.

have the scope to examine the mechanistic basis of the programming effect. It is widely believed that nutritional programming during early life is driven by changes to the epigenome [36]. There are a growing number of reports that maternal protein restriction can impact upon DNA methylation and histone modifications [37–39]. Although it was originally thought that the mitochondrial genome was not subjected to this level of control, it has recently been demonstrated that there is a mitochondrial DNA methyltransferase that can methylate cytosine residues [40]. Mitochondrial metabolism also determines the wider availability of S-adenosyl methionine for DNA methylation [41]. An alternative to epigenetic determination of maternally programmed effects involves overexposure of fetal tissues to glucocorticoids [11]. Undernutrition is known to suppress expression of placental 11β-hydroxysteroid dehydrogenase, allowing greater placental transfer of glucocorticoids from mother to fetus [42]. In sheep, early exposure to glucocorticoids upregulates expression of UCP2 [43]. Glucocorticoids may also impact upon DNA methylation [44].

4. Conclusion

This study has provided evidence that exposure to maternal undernutrition is associated with a decrease in mitochondrial respiration in kidneys of adult rats. Although the finding of increased expression of UCP2 mRNA could not be duplicated at the protein level, possibly reflecting changes in translation or turnover,results from these experiments suggest that early undernutrition may increase mitochondrial uncoupling under normal conditions, resulting in limited ability to effectively adjust respiratory function as cell mass declines with aging. The findings of the study are consistent with earlier reports that mitochondria may play a role in the early-life programming of adult disease. Further studies are required to assess the relative contribution of uncoupling to programming of physiological function in a broad range of tissues.

Authors' Contribution

S. Engeham was coinvestigator and was responsible for immunohistochemistry, histochemistry and assessment of mitochondrial DNA copy number. K. Mdaki measured mitochondrial respiration. K. Jewell performed western blots. R. Austin measured gene expression. A. Lehner was joint principal investigator and planned the functional studies. S. Langely-Evans was joint principal investigator, planned the studies, and wrote the paper.

Acknowledgments

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Research Article

The Effects of Prenatal Protein Restriction on β-Adrenergic Signalling of the Adult Rat Heart during Ischaemia Reperfusion

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A maternal low-protein diet (MLP) fed during pregnancy leads to hypertension in adult rat offspring. Hypertension is a major risk factor for ischaemic heart disease. This study examined the capacity of hearts from MLP-exposed offspring to recover from myocardial ischaemia-reperfusion (IR) and related this to cardiac expression of β-adrenergic receptors (β-AR) and their associated G proteins. Pregnant rats were fed control (CON) or MLP diets ($n = 12$ each group) throughout pregnancy. When aged 6 months, hearts from offspring underwent Langendorff cannulation to assess contractile function during baseline perfusion, 30 min ischemia and 60 min reperfusion. CON male hearts demonstrated impaired recovery in left ventricular pressure (LVP) and dP/dt_{max} ($P < 0.01$) during reperfusion when compared to MLP male hearts. Maternal diet had no effect on female hearts to recover from IR. MLP males exhibited greater membrane expression of β_2-AR following reperfusion and urinary excretion of noradrenaline and dopamine was lower in MLP and CON female rats versus CON males. In conclusion, the improved cardiac recovery in MLP male offspring following IR was attributed to greater membrane expression of β_2-AR and reduced noradrenaline and dopamine levels. In contrast, females exhibiting both decreased membrane expression of β_2-AR and catecholamine levels were protected from IR injury.

1. Introduction

Adult cardiovascular disease is associated with metabolic and physiological aberrations that occur during fetal development. Epidemiological studies provide evidence that intrauterine growth retardation increases the risk of hypertension and cardiovascular disease in adult life [1]. Such findings led to the fetal programming hypothesis that exposure to a suboptimal intrauterine environment can predispose to adult noncommunicable disease [2]. Animal models setup to test the hypothesis demonstrates the onset of cardiovascular pathologies by limiting fetal and neonatal growth through global maternal nutrient restriction [3], protein [4, 5], or micronutrient [6] restriction. The well-established maternal low-protein rat model produces offspring that develop elevated blood pressure which persists throughout adult life [7, 8]. Hypertension is a major independent risk factor for the development of ischemic cardiovascular conditions such as myocardial infarction and stroke [9]. Our laboratory has recently reported that male rats exposed to protein restriction *in utero* showed impaired cardiac contractile recovery to ischemia-reperfusion (IR) injury compared to offspring from control fed dams. Protein restriction had no effect on recovery of the female heart [10]. This sex-specific effect concurs with previous findings in rats in which intrauterine growth restriction as a result of either hypoxia or undernutrition caused cardiac remodelling and impaired recovery to IR in adult male offspring [11].

β-adrenergic signalling is key to cardiac contractile function and cardiac recovery following IR. The catecholamines adrenaline and noradrenaline activate myocardial cell surface β-adrenergic receptors. β_1 and β_2-AR are the principal β-receptor subtypes in the heart making up approximately 70–80% and 30–20% of the β-ARs expressed, respectively [12]. Adrenaline stimulates both β_1 and β_2-AR subtypes whereas noradrenaline acts mainly through β_1-AR [13]. β-AR activation involves release of the stimulatory Gαs subunit from the receptor. This stimulates adenylyl cyclase activity and

generation of cAMP- and PKA-mediated activation of the contraction-relaxation cycle [12, 14]. These effects are more tightly regulated following β_2-AR activation which acts in an antagonistic fashion by coupling Gαi leading to the attenuation of cAMP-mediated activation of PKA. Continual β-AR activation results in phosphorylation and receptor desensitisation through G protein uncoupling [12]. The receptors then translocate from the plasma membrane into vesicular cytosolic compartments and either targeted for degradation or dephosphorylation and recycled back to the membrane.

The interaction between IR injury and β-AR signalling in the heart has been widely investigated and established that myocardial ischaemia is commonly associated with an increase in membrane expression of β-ARs and that their overexpression prior to ischaemia increases IR-injury [15–19]. However, the effects of IR on β-AR signalling in developmental programming are yet to be explored. This study therefore aims to examine the capacity of isolated adult rat hearts from protein-restricted offspring to recover from IR injury in relation to alterations in shuttling of β-AR and G protein signalling between the sarcolemma and cytosolic compartments of the myocardium.

2. Methods

2.1. Animals. Twenty-four virgin Wistar rat dams (Harlan Ltd, Belton, Leicestershire, UK) weighing approximately 250 g were mated in the animal facilities at the University of Nottingham. The appearance of a semen plug on the cage floor confirmed that mating and rats were allocated either a control (CON) diet (180 g casein/kg, $n = 12$) or a low-protein diet (90 g casein/kg, $n = 12$, MLP), as described previously in detail [7]. The pregnant rats were then fed the isoenergetic semisynthetic diets throughout gestation until birth at 22 days of gestation. All mothers were then transferred to standard laboratory chow diet (B&K Universal Ltd, Hull, UK) and each litter culled to a maximum of 8 pups to minimise variation in the nutrition of the offspring during suckling. At 4 weeks of age, all offspring were weaned onto chow diet, to ensure that litters from control and low-protein fed rat dams only differed in terms of their prenatal nutrition. All experimental procedures were performed in accordance with the Home Office Guidance on the Operation of the Animals (Scientific Procedures) Act, 1986.

2.2. The Isolated Heart (Langendorff) Preparation. At 6 months of age, male and female rats from each CON and MLP litter were randomly selected ($n = 9$ or 10), anaesthetised using 3% isofluorane in 2 litres O_2/min and killed by cervical dislocation. The heart was rapidly excised and cannulated via the aorta to Langendorff perfusion apparatus within 90 seconds (AD Instruments, Oxford, UK) and perfused with Krebs Henseleit buffer (118 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, 11 mM glucose, and 1.25 mM $CaCl_2$ (pH 7.4)) bubbled with O_2-CO_2 (95 : 5, v/v) in a coronary retrograde fashion. Perfusion pressure was maintained at a constant pressure of 60 mmHg, with perfusate warmed to 37.4°C, and the heart immersed in a water-jacketed temperature-controlled glass chamber set at 37.4°C, therefore ensuring normothermia throughout the perfusion protocol. Contractile function was monitored by the careful insertion of a saline filled latex balloon (Linton Instruments) into the left ventricle which was adjusted to an end diastolic pressure of 5–10 mmHg. Left ventricular and perfusion pressure were continuously monitored through precalibrated physiological pressure transducers (Senso-Nor 844, AD Instruments). Data recording was not started until all variables were stable (15–30 min) after which the following 30 min were defined as baseline. Ischemia was then induced by switching off the coronary perfusion apparatus for a 30-minute period. Coronary perfusion was then reinstated for a further 60-minute period to assess cardiac responses during reperfusion. Data for left ventricular developed pressure (LVP), heart rate (HR) and left ventricular first derivative (dP/dt_{max}) were collected and processed using the Powerlab Data Acquisition System (AD) Instruments. To determine the effect of IR on myocardial expression of β-AR, hearts from CON or MLP offspring that underwent either (i) 30 min baseline perfusion alone ($n = 9$-10), (ii) 30 min baseline perfusion followed by 30 min ischemia ($n = 9$-10) or (iii) 30 min baseline perfusion followed by 30 min ischemia and 60 min reperfusion ($n = 9$-10) on the Langendorff apparatus were collected and snap frozen for western blot procedures.

2.3. Western Blot Analysis. Hearts collected for western blot analysis from the Langendorff study were used to prepare cardiac membrane and cytosolic fractionated samples as described by Fernandez-Twinn and colleagues [20]. Frozen whole heart tissue was ground to a powder in liquid nitrogen and homogenised briefly for 30 s in ice cold buffer containing 5 mM Tris pH 7.4, 2 mM EDTA, and protease inhibitor cocktail (Calbiochem). Homogenates underwent centrifugation at 1000× g for 15 min at 4°C to pellet nuclear material. The supernatant fraction was then spun at 100,000× g for 1 h 20 min at 4°C in an ultracentrifuge to generate a pellet and supernatant which corresponded to the plasma membrane and cytosolic fractions, respectively. The pellet was then resuspended in lysis buffer (50 mM HEPES pH 8, 150 mM NaCl, 1% Triton-X-100, 1 mM Na_3VO_4, 30 mM NaF, 10 mM $Na_4P_2O_7$, 10 mM EDTA, and protease inhibitor cocktail).

Protein concentrations of the supernatant and resuspended pellet fractions were determined using the Bio-Rad protein assay system (Bio-Rad, Hemel Hempstead, UK) according to the manufacturer's instructions. Samples were standardised to a concentration of 3 mg/mL with Laemmli's sample buffer (62.5 mM Tris pH 6.8, 2% SDS, 10% glycerol, 0.02% bromophenol blue, 150 mM dithiothreitol) and boiled for 3 min before equal protein quantities of each sample were separated by SDS PAGE. Proteins were transferred to nitrocellulose membrane (Hybond-C extra, Amersham Bioscience) for probing with antibodies to β_1-AR (Affinity Bioreagents; rabbit polyclonal raised against a synthetic peptide corresponding to residues 394–408 of mouse/rat β_1-AR), β_2-AR (Abcam; rabbit polyclonal raised against against residues

Table 1: Effect of maternal protein restriction on the cardiac recovery of male and female rat hearts during ischemia reperfusion. Measures of cardiac function include left ventricular pressure (LVP), the left ventricular first derivative (dP/dt_{max}), and heart rate (HR). These parameters were determined at baseline and during 60 min reperfusion following 30 min ischemia. Data are displayed as mean ± SEM values. Recovery 1 and 2 denote 0–30 and 31–60 min reperfusion periods, respectively.

	Males					Females				
	CON		MLP		*P*-value	CON		MLP		*P* value
	Mean	SE	Mean	SE		Mean	SE	Mean	SE	
LVP (mmHg)										
Baseline	44.67	3.0	50.18	5.3	0.38	43.74	4.1	50.57	5.1	0.31
Recovery 1	5.81	1.0	11.71	1.6	**0.01**	10.83	2.0	10.83	2.3	1.00
Recovery 2	7.25	1.7	15.33	3.3	**0.04**	11.31	2.3	13.24	2.6	0.58
Recovery 1 (% baseline)	13.8	3.3	23.31	4.0	0.09	28.41	6.3	25.16	6.6	0.88
Recovery 2 (% baseline)	18.3	5.3	33.24	8.0	0.16	29.33	6.6	31.11	7.2	0.87
HR (beats per min)										
Baseline	302.42	21.3	290.9	7.0	0.61	331.76	18.8	298.23	19.7	0.23
Recovery 1	112.16	26.5	105	24.4	0.85	129.53	25.1	183.46	26.9	0.16
Recovery 2	195.42	19.3	169.54	28.2	0.47	179.41	31.5	255.36	38.0	0.14
Recovery 1 (% baseline)	30.6	7.1	43.26	6.9	0.50	37.89	7.9	60.22	8.8	0.08
Recovery 2 (% baseline)	65.1	7.1	59.45	9.8	0.66	53.65	10.9	85.03	12.2	0.07
dP/dt (mmHg/s)										
Baseline	1186.01	101.9	1398.09	135.5	0.23	1252.42	106.5	1402.94	133.8	0.39
Recovery 1	381.59	32.9	625.55	78.1	**0.01**	515.82	66.5	530.37	53.4	0.87
Recovery 2	409.39	52.1	599.45	79.1	0.06	570.02	84.2	544.59	51.1	0.81
Recovery 1 (% baseline)	31.52	4.1	47.34	7.2	0.08	42.29	5.4	40.35	5.2	0.54
Recovery 2 (% baseline)	35.43	4.7	43.76	6.8	0.33	45.51	5.4	42.78	5.0	0.33

1–100 of human β_2-AR), G stimulatory protein (Santa Cruz Gαs (K-20): sc-823), and G inhibitory protein (Santa Cruz Gαi-3 (C10): sc262). Membranes were incubated in blocking solution (5% dried skimmed milk in TBS with 1% Tween 20) prior to incubation with primary antibodies. Horseradish peroxidise secondary antibody conjugated to rabbit IgG was used at a working concentration of 1 : 5000 (GE healthcare, Amersham, UK). Bands were developed on high performance chemiluminescence film (Hyperfilm ECL, Amersham) using ECL Plus reagent (GE healthcare, Amersham, UK). Densitometric analysis of band intensity across gels was performed using a Biorad Gel Doc XR imaging system and Quantity One 1D analysis software. Three replicates of a pooled extract were included on every gel and used as a standard to facilitate gel-to-gel comparisons.

2.4. Urine Collection and Catecholamine Assays. Rats at 5 weeks, 10 weeks, and 6 months of age were housed in metabolic cages for 24 hours and urine collected as previously described [21]. Urine concentrations of adrenaline, noradrenaline, and dopamine were determined using the DLD Diagnostika catecholamine ELISA kit (DLD Diagnostika GmbH, Hamburg, Germany), following the manufacturer's instructions.

2.5. Statistical Analysis. All results are presented as mean values with their standard errors. All data were analysed for homogeneity of variance, and if heterogeneous transformed by square root or logarithmic conversion. For the analyses, ischemia-reperfusion data were split into three distinct time bins: (1) 30 min baseline, (2) recovery 1 (0–30 min of reperfusion), and (3) recovery 2 (31–60 min of reperfusion). The effects of maternal diet on the recovery in LVP, HR, and dP/dt_{max} relative to baseline were analysed using a one-way repeated measure ANOVA. Western blot data were analysed by three-way ANOVA considering maternal diet, sex, and ischemia-reperfusion treatment as fixed factors. ELISA data were analysed by three-way ANOVA considering maternal diet, sex, and age as fixed factors. Measurements from related animals from the same litter were grouped in all ANOVA analyses and assigned as a random factor. All statistical analyses were performed using SPSS version 18. In all analyses, $P < 0.05$ was considered statistically significant.

3. Results

3.1. Effect of Maternal Diet on Functional Recovery of Isolated Hearts to IR Injury. The effect of maternal diet on the recovery of contractile function relative to baseline in the first (R1) and second (R2) 30 min periods of reperfusion was measured in Langendorff-perfused hearts from male and female rats. Maternal diet had no effect on LVP, HR, or dP/dt_{max} baseline indices in either sex (Table 1). Similarly, there was no significant effect of maternal diet on the recovery of female

indices of cardiac function (LVP, dP/dt_{max}, HR) to ischemia-reperfusion insult (Table 1). However, significant differences in the capacity of male hearts to recover from ischemia-reperfusion injury were observed between CON- and MLP-exposed offspring. Interestingly the recovery of LVP in MLP offspring during R1 and R2 phases of reperfusion was 2-fold higher than the recovery seen in CON males ($P < 0.01$) (Table 1). A similar result was observed with dP/dt_{max} where MLP males exhibited almost a 2-fold greater recovery during the R1 phase ($P < 0.05$) and a trend ($P = 0.06$) for an improved recovery during the R2 phase in comparison to CON hearts (Table 1). In contrast, there was no significant effect of maternal diet on recovery of heart rate (Table 1).

3.2. β Adrenergic Signalling Response to IR Injury in the Isolated Rat Heart. β adrenergic signalling is a central mechanism involved in the maintenance of cardiac inotropic and chronotropic function and has been shown to play a key role in recovery of the heart from IR. Accordingly, we quantified levels of the two main β-AR isoforms, β_1-AR and β_2-AR, as well as levels of the stimulatory and inhibitory G proteins Gαs and Gαi, respectively, in isolated hearts exposed either to baseline perfusion (B), baseline perfusion plus ischemia (I), or baseline perfusion plus ischemia plus reperfusion (IR). Expression was measured in both membrane (Figure 1) and cytosolic (Figure 2) fractions prepared from each heart. Baseline membrane or cystolic expression of all proteins was not significantly altered by gender or maternal diet; however differential levels of expression were observed following ischemia and ischemia-reperfusion.

β_1-AR levels at the membrane increased following ischemia and returned to baseline levels by the end of 60 min reperfusion (Table 1; $P < 0.05$). This correlated with a decline in β_1-AR in the cytosol by the end of the ischemic period which had declined further by the end of reperfusion (Figure 2(a); $P < 0.001$). A significant interaction between diet and sex ($P < 0.05$) was also observed for membrane expression of β_1-AR indicating that the overall level of receptor expression in females was lower in the MLP group when compared to CON, an effect not observed in males.

Cardiac membrane expression of β_2-AR revealed a significant interaction between maternal diet, sex, and ischemia-reperfusion treatment (Figure 1(b); $P < 0.05$). β_2-AR decreased in CON females following ischemia which was in complete contrast to all other groups where receptor expression levels were unchanged from baseline levels. Following reperfusion, β_2-AR expression in CON females remained unchanged from levels observed after ischemia. Hearts from CON males showed decreased membrane expression of β_2-AR after reperfusion similar to the decreased expression observed in CON female hearts following ischemia (Figure 1(b)). However, hearts from MLP females and males maintained baseline levels of β_2-AR expression throughout ischemia and reperfusion.

β_2-AR expression in cardiac cytosolic fractions was found to increase after ischemia and increased further following reperfusion (Figure 2(b); $P < 0.001$) which was in direct contrast to β_1-AR cytosolic expression. These findings suggest that ischemia-reperfusion initiates a differential response in membrane trafficking of β_1-AR and β_2-AR from the cytosol to the cardiac membrane. Despite changes in β-AR isoform within the cardiac membrane and cytosol, no overt changes were observed in the levels of Gs and Gi (Figure 1(d) and Figure 2(d)) proteins within either of these cellular locations.

3.3. Urinary Catecholamine Levels in the Rat at 5 Weeks, 10 Weeks, and 6 Months of Age. Concentrations of the catecholamines adrenaline, noradrenaline, and dopamine were measured in the urine of rats at 5 weeks, 10 weeks, and 6 months of age. No significant differences were observed in the concentration of adrenaline at each stage of development (Table 2). Urine levels of noradrenaline however were significantly affected by maternal diet where greater levels were observed in CON compared to MLP rats (Table 2; $P < 0.02$). In addition, a significant effect of age was observed where noradrenaline concentrations were significantly higher at 5 and 10 weeks of age respectively ($P < 0.02$). An interaction between age and sex was also evident ($P < 0.01$) where urine concentrations of noradrenaline in females were highest at 5 weeks of age with a concentration of 15 ng/mL before dropping to 8 ng/mL at 10 weeks of age, and remaining at this level when aged 6 months. For males, noradrenaline concentration at 10 weeks of age had risen from 10 ng/mL observed at 5 weeks to 14 ng/mL before dropping by 60% to 8 ng/mL at 6 months of age.

Dopamine concentrations were significantly reduced by an MLP diet (Table 2; $P < 0.01$), and an interaction between sex and age was also observed (Table 2; $P < 0.04$). Female rats had a significantly reduced urinary excretion of dopamine at 6 months of age compared to both 5 and 10 weeks of age. In contrast, urinary excretion of dopamine in male rats increased significantly with age. At 6 months of dopamine concentrations were significantly higher than the level determined at 5 and 10 weeks of age.

4. Discussion

The key finding from the present study is that hearts from MLP male offspring demonstrated improved recovery in cardiac function following IR when compared to CON males, whereas IR recovery in females was unaffected by maternal diet. This study verifies that sex is an important factor in the capacity of the heart to recover from IR injury [10, 15, 22–24]. This contrasts starkly with our previous finding where CON male rats demonstrated improved contractile recovery to IR compared to CON females or MLP rats of either sex [10]. This discrepancy may be explained through experimental differences. In the initial study, rats were restraint for intraperitoneal injections and subject to restraint stress. This procedure was absent in the present study. Introduction of restraint prior to Langendorff perfusion may have conditioned the CON male rat hearts' response to stress and enhanced their capacity to recover from IR injury. Stressors, such as hyperthermia, hypo- and hyperoxia, and dexamethasone treatment, have been observed to precondition and protect hearts from subsequent

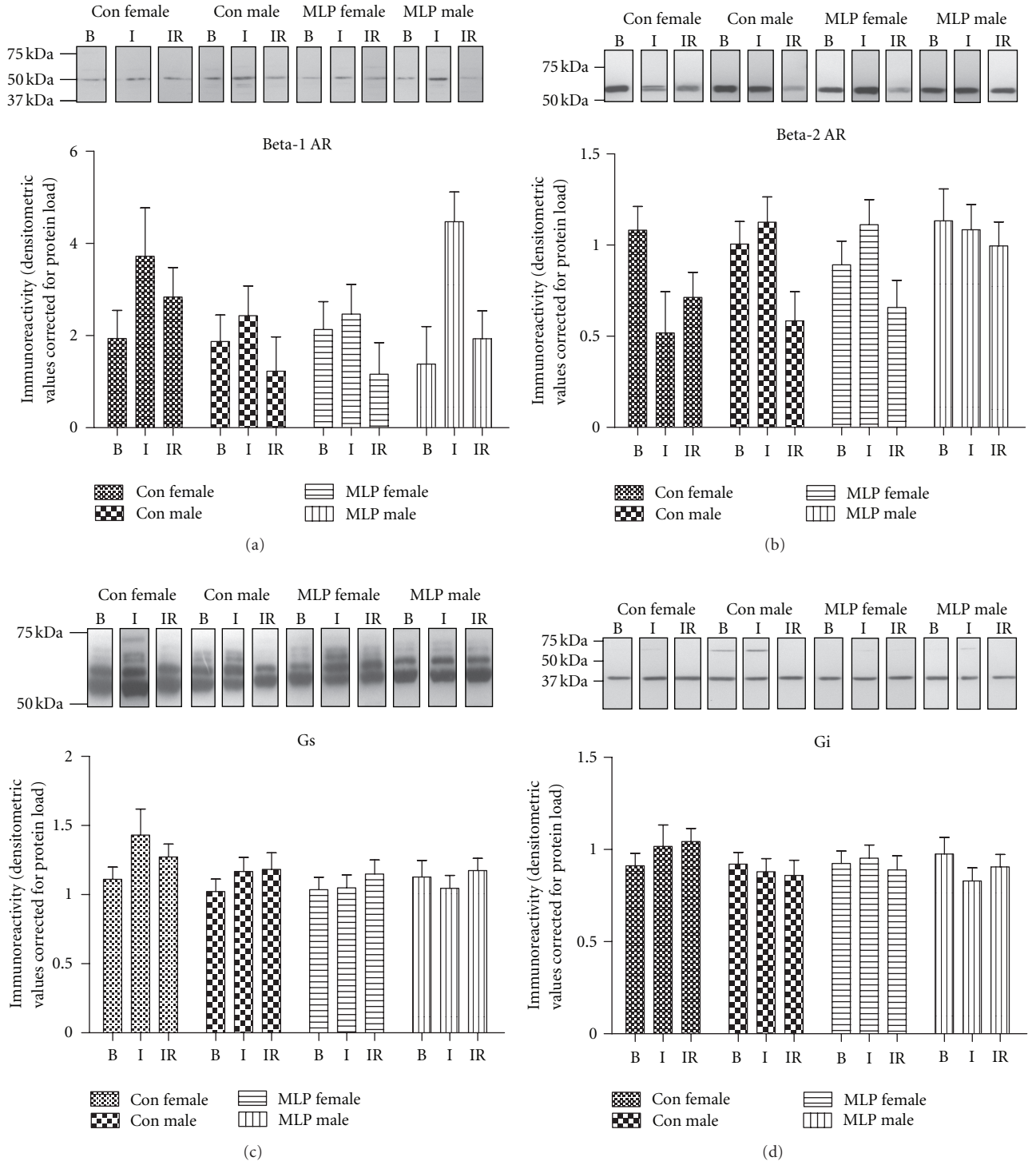

Figure 1: β-adrenergic receptor (AR) isoforms, Gs, and Gi protein expression in cardiac membranes from CON and MLP Langendorff perfused rat hearts at baseline and following ischemia and ischemia reperfusion. Cardiac membrane expression of β_1-AR (a), β_2-AR, (b) Gs (c), and Gi (d) was determined by western blot analysis. Data were expressed as mean ± SEM values for isolated hearts exposed to either baseline (B) perfusion, 30 min ischemia (I), or 30 min ischemia followed by 60 min reperfusion (IR) from Con Female, Con Male, or protein-restricted (MLP) MLP Female or MLP Male rats. Data were analysed by three-way ANOVA. β_1-AR showed an interaction effect between maternal diet and sex ($P < 0.05$) and an effect of ischemia reperfusion treatment ($P < 0.01$). β_2-AR expression showed an interaction effect of maternal diet, sex, and ischemia reperfusion treatment ($P < 0.05$).

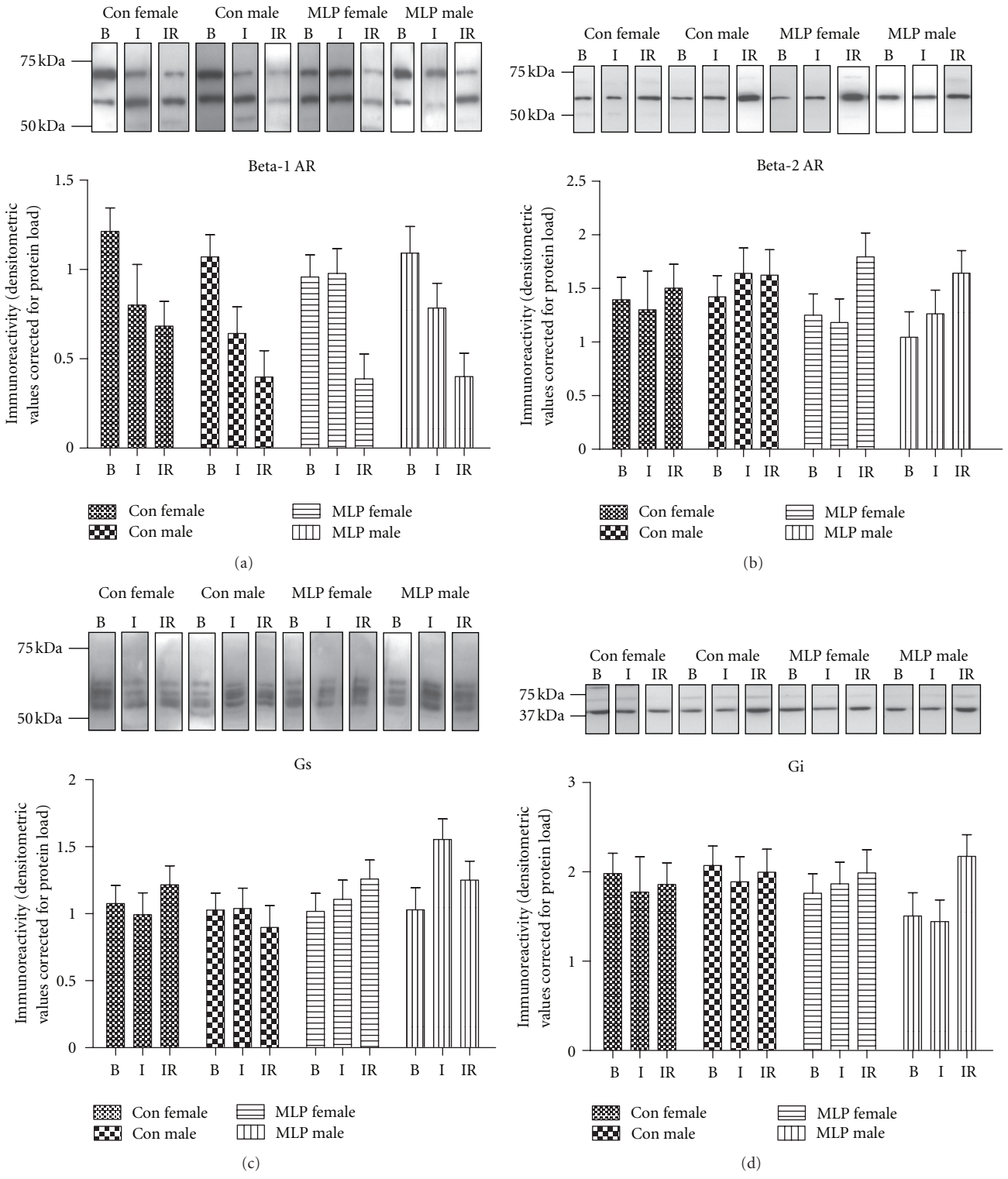

Figure 2: β-adrenergic receptor (AR) isoforms, Gs, and Gi protein expression in cardiac cytosolic fractions from CON and MLP Langendorff perfused rat hearts at baseline and following ischemia and ischemia reperfusion. Cardiac cytosolic expression of β_1-AR (a), β_2-AR (b), Gs (c), and Gi (d) was determined by western blot analysis. Data were expressed as mean ± SEM values for isolated hearts exposed to either baseline (B) perfusion, 30 min ischemia (I) or 30 min ischemia followed by 60 min reperfusion (IR) from Con Female, Con Male, or protein-restricted (MLP) MLP Female or MLP Male rats. Data were analysed by three-way ANOVA. β_1-AR and β_2-AR showed effects of ischemia reperfusion treatment ($P < 0.001$).

Table 2: Catecholamine concentration levels in the urine of rats at 5 weeks, 10 weeks and 6 months of age. Urine was collected over a 24 h period and the concentration (ng/mL) of adrenaline, noradrenaline and dopamine were measured by ELISA. Adrenaline and dopamine were log transformed and Noradrenaline underwent square root transformation to produce homogeneous data. The transformed data was then analysed by three-way ANOVA and expressed as mean and SEM with corresponding P values for each analysis.

		Adrenaline (ng/mL)		Noradrenaline (ng/mL)		Dopamine (ng/mL)	
		Mean	SEM	Mean	SEM	Mean	SEM
CON Female	5 weeks	7.72	3.23	249.19	57.05	316.14	69.9
	10 weeks	23.01	4.27	128.28	75.48	294.8	83.17
	6 months	18.96	4.27	151.38	61.63	113.37	43.2
MLP Female	5 weeks	6.88	3.82	240.61	53.37	158.64	55.66
	10 weeks	14.40	3.49	51.71	75.48	107.61	29.13
	6 months	14.57	3.02	40.62	67.51	103.9	24.4
CON Male	5 weeks	6.94	3.02	177.08	57.05	116.99	14.35
	10 weeks	11.17	4.93	374.06	61.63	284.9	56.35
	6 months	12.63	3.49	59.58	75.48	276.2	72.5
MLP Male	5 weeks	8.59	4.27	82.80	61.63	163.98	61.4
	10 weeks	8.31	3.49	147.51	67.51	133.11	35.5
	6 months	8.22	3.23	98.86	61.63	148.1	56.5
ANOVA		P-values		P-values		P-values	
Diet		0.117		**0.016**		**0.010**	
Sex		0.115		0.906		0.559	
Age		0.072		**0.022**		0.400	
Diet × Sex		0.662		0.842		0.880	
Diet × Age		0.171		0.623		0.353	
Sex × Age		0.438		**0.011**		**0.038**	
Diet × Sex × Age		0.768		0.105		0.222	

ischemic injury [25–28]. If true, hearts from either CON females or MLP of either sex were not receptive to preconditioning. This is consistent with the cardioprotective effects of ischemic preconditioning only observed in male and not female mouse hearts [29]. Interestingly, there was no significant difference in cardiac recovery following ischemia in MLP rats between the present and previous study. This suggests that maternal protein restriction may block the positive effects of preconditioning. MLP rats consistently develop elevated blood pressure which persists throughout adulthood and may prevent cardiac preconditioning in these animals. Indeed evidence suggests that hypertensive individuals hearts are not susceptible to preconditioning [30]. Previous work has shown that protein restriction programmes changes in the hypothalamic-pituitary-adrenal axis, which is likey to have a major impact on the response of MLP rats to stress [31].

To investigate how cardiac function during IR relates to β-AR signaling, we determined protein expression of the β-AR isoforms, and the Gs and Gi proteins in baseline-perfused ischemia or ischemia-reperfused hearts. β_1-AR expression was associated with increased expression within the plasma membrane after ischemia that returned to baseline levels following reperfusion. This suggests that the β_1-AR expression is upregulated in the membrane in response to ischemia and declines throughout reperfusion. This agrees with Strasser et al. [19], who identified that sarcolemmal β_1-AR levels increase following 50 min ischemia that was associated with decreased adenylyl cyclase activity. More recently, β_1-AR protein expression in isolated rat hearts has also been demonstrated to increase at the plasma membrane following 30 min ischemia but with no change in adenylyl cyclase activity [32]. Further agreement with this study is that β_1-AR density at the plasma membrane decreases following 60 min reperfusion compared to levels after ischemia which was associated with a doubling in adenylyl cyclase activity [32]. It is important to concede that the changes in β_1-AR expression following ischemia and reperfusion in the present study may not be reflective of downstream changes at the level of adenylyl cyclase.

Protein expression of β_2-AR in the cardiac membrane decreased following ischaemia in CON females and following reperfusion in CON males and MLP females. These findings demonstrate subtle sex-specific responses in downregulation of β_2-AR at the cardiac membrane to ischaemia and reperfusion. To our knowledge, this is the first time sex-specific effects of IR on β_2-AR abundance have been reported. A previous study focussing solely on male hearts reported that β_2-AR density at the plasma membrane was unaffected following 30 min ischemia but decreased with 60 min reperfusion, thus agreeing with the present findings in CON males [32]. In contrast β_2-AR protein expression in the

cardiac membrane of MLP males did not change throughout ischemia and reperfusion from baseline levels. Consequently, β_2-AR levels were higher in MLP males compared to all other treatment groups following reperfusion.

All these changes in BAR expression at the membrane occurred against a background where neither Gs nor Gi expression levels changed, suggesting that β-AR density rather than G protein content at the cardiac membrane is the rate limiting component mediating cardiac contractility. Alterations in expression of β-AR isoforms through transgenic mouse models show profound effects on haemodynamic function of the heart [15, 33–35]. Recent work has focussed on the role of β_2 rather than the β_1-AR activation in cardiac pathology due to its specific capacity to enhance Ca^{2+} homeostasis and protection against apoptosis [35–38]. However, transgenic mouse models have shown that these therapeutic effects cannot be recapitulated by simply overexpressing β_2-AR of the heart [16, 34, 35]. Despite increased inotropic and chronotropic activity, cardiac β_2-AR overexpression led to progressive cardiac hypertrophy and delayed onset cardiomyopathy. Moreover, β_2-AR overexpression exacerbated ischemic injury but in the hearts of male mice only [15]. Female mice overexpressing β_2-AR were protected from damage following IR and linked to the female sex hormone oestrogen acting through endothelial nitric oxide synthase to prevent Ca^{2+} dysregulation in the tissue [15]. Such findings suggest that the contractile function of the female heart is less sensitive to β_2-AR expression changes. This may explain why cardiac β-AR expression levels observed between CON and MLP groups in the present study resulted in differences in haemodynamic parameters in male rats only.

The cardiac pathology observed in transgenic mouse models is possibly an artefact of such high β_2-AR overexpression and does not represent a moderate increase in β_2-AR expression [34]. Comparisons of β_2-AR overexpression have shown that 100–360-fold increases in β_2-AR expression resulted in the onset of cardiomyopathy, whereas a moderate 60-fold increase had no overt pathological consequence [34]. Indeed recent studies have presented evidence supporting the putative protective role of β_2-AR signalling against cardiac pathology [37–39]. Long-term β_2-AR agonist treatment in conjunction with a β_1-AR blocker was found to improve survival and contractility in a rat model of dilated cardiomyopathy [36, 37]. Furthermore treatment with the β_2-AR agonist clenbuterol alone was sufficient to exert a cardioprotective effect in rat hearts through a reduction in myocardial apoptosis and oxidative stress and improving diastolic function following IR injury [38]. This cardioprotective effect of β_2-AR activation is thought to occur via stimulation of Gi rather than Gs-mediated signalling mechanisms which enable Ca^{2+} regulation to be maintained and apoptosis to be inhibited.

Our key finding is that MLP male hearts do not downregulate β_2-AR protein expression within the cardiac membrane following IR, which is in complete contrast to CON males. We propose that downregulation of β_2-AR in response to IR is the cause of impaired contractile recovery in CON male hearts. Cardiac membrane expression of β_2-AR also decreased after reperfusion in CON and MLP female hearts to a level comparable to CON males but despite this decrease showed no impairment in contractile recovery following ischemia. This may result from the protective role of oestrogen in maintaining a favourable Ca^{2+} microenvironment within female hearts during reperfusion that limits tissue damage and counteracts any changes in β-adrenergic signalling activity that would otherwise impair contractile recovery after ischemia. Measurements of urinary catecholamine levels indicate that by 6 months of age there is no overt difference in either adrenaline or noradrenaline levels in these animals. However, we did observe an increase in dopamine levels in males compared to female rats at 6 months of age. It is not clear how this difference affects cardiac function of these hearts. Dopamine is routinely administered as a therapy in heart failure to improve cardiac function [39]. This is thought to occur primarily through activation of β-ARs in the heart. We did not see any evidence to indicate that this sex-specific difference in dopamine had any impact on basal levels of expression of any of the β-adrenergic signalling components measured in this study or on baseline activity of the hearts prior to ischemic reperfusion. Further work is required to explore other potential receptor targets of dopamine which may alter cardiac function in response to ischemia reperfusion.

Urine excretion of noradrenaline and dopamine was significantly lower in MLP compared to CON animals and may offset the ischaemic damage observed in CON males. Evidence to support this is the fact that depletion of catecholamines by surgical denervation causes a redistribution of β-ARs, decreasing β_1 and increasing the β_2 subtype although total β-AR content remained the same [40]. Furthermore, suppression of noradrenaline turnover in the rat heart protects against myocardial IR injury [41].

5. Conclusion

We propose that the contractile dysfunction in male CON hearts following IR occurs as a result of increased catecholamine production and decreased β_2-AR expression at the cardiac membrane. Likewise, protein expression of β_2-AR at the cardiac membrane decreased in response to IR in female CON and MLP hearts. However they displayed an improved cardiac recovery compared to CON males suggesting specific protective mechanisms in female hearts that counteract alterations in β-adrenergic receptor expression. Our findings indicate that in male offspring prenatal protein restriction maintains cardiac membrane expression of β_2-AR during IR which improves cardiac contractile recovery. In contrast, female hearts were resistant to these effects suggesting subtle sex differences in the molecular mechanisms controlling cardiac membrane expression of β-AR in response to IR.

Conflict of Interests

The authors declare that they have no conflict of interests.

Acknowledgments

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Review Article

Antenatal Corticosteroids: A Risk Factor for the Development of Chronic Disease

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Preterm birth remains a major health issue worldwide. Since the 1990s, women at risk for preterm birth received a single course of exogenous antenatal corticosteroids (ACSs) to facilitate fetal lung maturity. More recently, repeated or multiple courses of ACS have been supported to provide continued fetal maturity support for women with continued risk of preterm birth. However, exogenous ACS reduces birth weight which, in turn, is associated with adverse adult outcomes such as coronary heart disease, stroke, hypertension, and type 2 diabetes. The long-term effects of ACS exposure on HPA axis activity and neurological function are well documented in animal studies, and it appears that ACS, regardless of dose exposure, is capable of affecting fetal HPA axis development causing permanent changes in the HPA axis that persists through life and is manifested by chronic illness and behavioral changes. The challenge in human studies is to demonstrate whether an intervention such as ACS administration in pregnancy contributes to developmental programming and how this is manifested in later life.

1. Introduction

The "developmental origins of health and disease" (DOHaD) refers to a concept where exposure to environmental factors such as maternal nutrition, body composition, and stress hormone levels sends signals to a developing fetus that potentially influences the metabolic phenotype of the offspring and its risk of chronic diseases later in life [1–5]. This concept was originally developed to explain observations between the high rates of death due to coronary heart disease in areas of England and Wales with high neonatal mortality and proposed that intrauterine deprivation was an important mediator [6]. Further studies found an inverse relationship between birthweight and coronary heart disease [7]. The association of low birth weight and increased incidence of chronic illnesses of adulthood, such as heart disease, hypertension, type 2 diabetes, has been documented in numerous studies [8–12]. Low birth weight has been proposed as an indicator of an environmental adversity during fetal development and may suggest fetal processes or programming are in place. Two mechanistic hypotheses have been proposed to explain how fetal programming may arise: fetal malnutrition and fetal overexposure to glucocorticoids both of which may have effects either directly or indirectly upon the developing fetus. Exposure to excess glucocorticoids in utero acts to program the fetal hypothalamic-pituitary-adrenal (HPA) axis, permanently altering basal and stress-induced HPA axis activity and regulation in the offspring throughout life. Fetal exposure to excess glucocorticoids can occur via a number of mechanisms such as maternal stress during pregnancy and maternal treatment with synthetic glucocorticoids (sGC), more commonly known as antenatal corticosteroids (ACS).

This paper will focus on ACS and its effect on the developing HPA axis and building the case for the use of ACS as a risk factor for the development of chronic disease in the offspring.

2. Development and Function of the Hypothalamic-Pituitary-Adrenal (HPA) Axis

Activation of the HPA axis after exposure to a stressor is part of a normal adaptive response allowing the organism to respond to changes in its environment [13]. Stress results

in activation of central neurocircuitry, which in turn signals the hypothalamus to produce and secrete corticotrophin-releasing hormone (CRH) and vasopressin (AVP). CRH and AVP lead to the release of adrenocorticotrophic hormone (ACTH) from the anterior pituitary which stimulates the production and secretion of glucocorticoids (GC), principally cortisol in humans. Cortisol binds the glucocorticoid receptors (GR) in multiple target tissues to maintain homeostasis following stress. Cortisol also binds to GR and mineralocorticoid receptors (MR) in the limbic regions (including hippocampus and prefrontal cortex) to modify behaviours and learning and memory. In addition, cortisol acts to feedback and decrease HPA function in a classic endocrine feedback loop. Tightly regulated feedback control of cortisol is critical as prolonged tissue exposure to GC leads to metabolic and behavioural disorders [14]. The timing of maturation of the HPA axis relative to birth is highly species-specific and is linked to landmarks of brain development. In humans, like animals that give birth to mature young (primates, sheep, and guinea pigs), maximal brain growth and a large proportion of neuroendocrine maturation takes place *in utero* [15, 16].

3. Role of 11β-HSD-2

Low levels of GC are critical for normal fetal development; however, fetal exposure to elevated glucocorticoids can inhibit growth and adversely impact brain development. Under normal conditions, access of maternal cortisol to the fetus is low due to the placental expression of the enzyme, 11β-hydroxysteroid dehydrogenase-2 (11β-HSD-2) [17]. Placental 11β-HSD-2 transforms cortisol into its much less active 11-keto form, cortisone. This protective placental enzymatic barrier is very efficient, such that only 10–20% of maternal cortisol crosses the placenta and reaches the fetus. This 10–20% passage of active maternal GC to the fetus reflects an anatomical bypass of the enzyme, presumably adding to the provision of GC for normal key developmental processes in the fetuses such as maturation of the lung and other fetal organs [18].

Low levels of placental 11β-HSD-2 activity have been correlated with low birth weight in humans and rodent models [19–22]. Reduced levels of 11β-HSD-2 may contribute to greater signaling within the placenta itself which may impact fetal development by altering placental function. Rodent studies have demonstrated that variations in placental 11β-HSD-2 levels have correlated with altered expression in glucose and amino acid transporter molecules as well as growth factors in the placenta [23].

It is clear that placental 11β-HSD-2 plays a key role in mediating the flow of environmental signals to the fetus. Animal studies have demonstrated that prenatal stress can lead to a reduction in placental 11β-HSD-2 activity suggesting then that the fetus and placenta are exposed to extra excessive amounts of GC [24]. In addition, dietary protein restriction selectively reduces 11β-HSD-2 activity suggesting a common mechanism by which malnutrition and excess GC can programme adult biology [25, 26].

Unlike endogenous GC, the synthetic GCs utilized as ACS (dexamethasone and betamethasone) are poor substrates for 11β-HSD-2 and readily passes the placenta barrier. This lack of restriction allows ACS to exert its effect in the same manner as excessive endogenous GC.

4. Other Stress-Related Programming Mediators

While it is clearly evident the role GC has on influencing development under normal and stress-related circumstances, other substances are released by the mother and fetus in response to stress. Catecholamines, in particular adrenaline and noradrenaline, are of importance since they are both released by stress and may influence placental function and also transport across the placenta to the fetus [27]. However, more exploration in this area is required to determine how these catecholamines function in normal and abnormal situation and what contribution they may play in enhancing disease risk.

5. Use of Antenatal Corticosteroids (ACSs) in the Management of Women at Risk for Preterm Birth

Preterm delivery affects over 7–12% of births in North America alone and is responsible for up to 75% of neonatal deaths [28, 29]. Despite advances in medical technology, the prevalence of preterm birth worldwide is increasing [29].

Respiratory distress syndrome, as a consequence of immature lung development, is a significant risk of preterm birth and the major cause of early neonatal mortality and morbidity [30]. Infants born very preterm (less than 32 weeks of gestation) often require respiratory support, in the form of positive pressure ventilation and prolonged oxygen support. A substantial proportion have intraventricular haemorrhages and associated "white matter brain injury" (grade 3 or 4 intraventricular haemorrhages, periventricular leukomalacia), bronchopulmonary dysplasia, and severe retinopathy of prematurity, all of which contribute significantly independently and combined to an adverse neurodevelopmental outcome in later life [31, 32]. Infants born preterm who survive have an increased risk of hospitalizations [33]. The personal and emotional costs for affected individuals and their families are high, as are the immediate and long-term monetary costs of these morbidities for parents and society [33]. Thus, preterm birth and preterm infants continue to remain a significant health issue worldwide.

The immature fetal lung is the target organ when women at risk of preterm birth are given ACS. Stimulation of the pulmonary surfactant system has been regarded as the most important effect of ACS. In addition, ACS can alter lung fluid absorption and alveolar development by inducing genes associated with the synthesis of surfactant proteins, fatty acid syntheses, the epithelial sodium channel, and the membrane protein sodium/potassium ATPase [34, 35]. The enhancement of these mechanisms causes an accelerated

maturation of the fetal lung and reduces the severity of respiratory distress syndrome (RDS) in the first few days after a preterm birth. This, in turn, contributes to a reduction in mortality and other neonatal morbidities associated with preterm birth. In 1972, Liggins and Howie published the results of the first RCT evaluating the effects of a single course of ACS [36]. Among those women who had been in spontaneous preterm labour, ACS reduced the risk of respiratory distress syndrome (RDS) (9.0% versus 25.8%, $P = 0.003$) and early neonatal mortality (3.2% versus 15.0%, $P = 0.01$). Over the subsequent 20 years, 12 additional RCT, involving over 3000 infants, showed a benefit of a single course of ACS with no adverse effects [37].

Since the early 1990s, the recommendation has been that women, between 24 and 34 weeks of gestation and at high risk of preterm birth, receive a single course of ACS (2 doses of ACS 12 hours apart) [38, 39]. However, approximately 50% of women given a first course of ACS remain undelivered 7–14 days later, and, for these women, the question had arisen as to whether repeated courses of ACS should be given [40]. There has been no consensus on repeated courses of ACS in the management of women at risk for preterm birth [41]. In the past decade, 10 trials have been conducted to evaluate the risks and benefits of repeated courses of ACS to women at risk of preterm birth. The most recent review in the Cochrane Database of these trials involving over 4730 women and 5650 infants concludes that repeat courses of ACS does reduce the risk of RDS (risk ratio (RR) 0.83, 95% confidence interval (CI) 0.75–0.91), and serious infant outcomes (RR 0.84, 95% CI 0.75–0.94) compared to no repeat ACS treatment [42]. However, these benefits were associated with a significant reduction in size at birth. Because of this reduction in size at birth, concern over the long-term effects in the offspring from repeated courses of ACS has been raised, and more research on the long-term effects has been recommended [43].

6. Long-Term Clinical Significance of ACS Exposure in the Offspring

Dalziel et al. reported on the cohort in the initial trial by Liggins and did not identify overt neurological and physiological effects of a single course of ACS in late gestation but did identify early markers of insulin resistance [44, 45]. Retrospective studies evaluating the effect of repeated courses on neonates and children have been somewhat conflicting; while they suggest benefit in terms of lung function in the neonatal period, there appears to be an increased risk of other outcomes, namely, long-term effects on behavior [46–49]. The 2-year followup of the recent RCT evaluating the use of repeated courses of ACS has reported no significant differences in the rate of deaths or major neurodevelopmental difficulties at 2 years of age [42, 50–54]. However, caution must be exercised because measurements at 2 years of age have limited predictive abilities and are only moderately correlated with developmental outcomes at later ages [55]. In addition, these outcome studies did not address the question of potential programming effect from ACS and impact on chronic illness development. Several ongoing studies are currently taking place to evaluate the children closer to school age which include the Canadian Institutes of Health Research (CIHR)- funded Multiple Antenatal Corticosteroids Study 5-year followup (MACS-5) and the 6-year evaluation of the ACTORDS participants [56]. MACS-5 is specifically designed to evaluate the effect of ACS on the developing brain, specifically the hippocampus, by assessing memory, attention, and behaviour as well as neurocognitive abilities; the ACTORDS study has focused on neurocognition in addition to other developmental abilities as well as early biomarkers for chronic illness [56].

7. Potential Effects of ACS during Pregnancy

Much of what is known about the effect of ACS on various structures is from animal studies.

7.1. Brain Development. Endogenous GC is important for normal development of the central nervous system; circulating levels of GC are maintained at very low levels throughout the majority of gestation [57, 58]. However, levels will change to meet the needs of the developing brain during "critical windows." Studies have shown that 11β-HSD-2 in the fetal human brain is silenced between 19 and 26 weeks which would lead to localized increases in GC signaling [59, 60]. GC is necessary for neuronal maturation, remodeling of axons and dendrite and cell survival [61, 62]. However, sustained elevation or depletion of GC during these "critical windows" of fetal development can modify brain structure with significant consequences to future function [63, 64]. Prenatal GC administration has been shown to retard brain weight at birth in sheep [65], as well as delay maturation of neurons, myelination, glia, and vasculature [60]. It has been shown to have widespread effect on neuronal structure and synapse formation and altered hippocampal structure and volume which can affect memory and attention [60, 65–68].

The hippocampus highly expresses GR and MR and, as such, is vulnerable to high sustained levels of GC. Rodent models show that prenatal stress decreases synaptic spine density and neurogenesis which correlates with deficits in cognition and learning. Similar effects are seen in primates (baboons and rhesus monkeys) where exposure to dexamethasone or betamethasone leads to reduced levels of neuritogenesis and neuronal plasticity and pronounced degeneration of areas within the hippocampus. These effects were dose associated suggesting multiple exposures induced more severe damage that a single injection [68–72].

7.2. HPA Axis. A number of animal models have shown that maternal administration of sGC during pregnancy has profound acute and long-term effects on the developing HPA axis. The fetal brain contains high levels of GR over the 2nd and 3rd trimesters, with highest levels in the limbic system (which includes prefrontal cortex, hippocampus and amygdala), the hypothalamus paraventricular nucleus (PVN; source of CRH and AVP), and the anterior pituitary (site of ACTH production) [73]. Exposure of these structures

to high levels of GC can lead to permanent programming of the function of these structures. This "programming" takes place via modification of both the GR and MR levels in the hippocampus, hypothalamus, and pituitary gland. Prenatal exposure to sGC leads to permanent changes in the expression of GRs and MRs in these structures resulting in altered negative feedback sensitivity and altered set points for HPA function. Long-term changes in the regulation of HPA function can predispose to chronic cardiometabolic and neurological disorders. In addition, imbalance of GR and MR in the limbic system can have profound effects on behaviours, including learning and attention [74].

The effects of sGC are also noted to be sex dependent [75, 76]. Administration of a single course of sGC in a guinea pig model in a dose and regimen comparable to that used in humans demonstrated significantly increased levels of MR and GR in the brains of female-exposed guinea pigs. Multiple course exposure to sGC in the same model resulted in dose-dependent reductions in hippocampal NMDA-receptors in the hippocampus and modification of hippocampal long-term potentiation (LTP; the biological substrate of learning and memory), in female but not male offspring [77, 78].

Limited studies have been undertaken to establish the long-term effects of fetal exposure to sGC on the HPA axis function in nonhuman primates. Uno et al. [79] and de Vries et al. [80] were both able to demonstrate that repeated exposure to sGC in rhesus monkeys and Vervet monkeys resulted in offspring demonstrating elevated cortisol levels and altered responses to stress throughout life.

8. Mechanisms of Programming for ACS

A key aspect of the DOHaD hypothesis is that the effects of the environment are mediated by physiological and metabolic effects during fetal and early postnatal life [1–5]. The HPA axis is a central mediator of the programming process. Prenatal stress leads to numerous cardiovascular and endocrine changes in the mother, including increases in plasma ACTH, β-endorphin, cortisol, and catecholamine concentrations. Although the placenta acts as a barrier to many of these maternal factors, a number will still pass to the fetus because of potential attenuation of 11-β-HSD-2 in noncritical windows. There may be activation of the fetal sympathetic nervous system which will contribute to programming of the HPA axis and lead to an altered physiological response in the fetus. Administration of ACS can be seen as mimicking "*prenatal stress.*" Its effects are more direct as it is able to bypass the functional barrier of placental 11-β-HSD-2 and bind directly to the GR and MR in the vulnerable regions of the hippocampus and alter HPA axis activity. ACS has been shown from the clinical trials to affect in utero weight gain and lead to a reduced birth weight [42], a surrogate crude measure which has been associated with the development of hypertension, type 2 diabetes, and cardiovascular disease in adulthood [81].

8.1. Role of Epigenetics. There is evidence that the early environment can permanently influence the genome through epigenetic mechanisms and in this way modifies endocrine function, metabolism, and behaviour of offspring [82–87]. Gene expression can be epigenetically modified through alterations in DNA methylation and chromatin structure (i.e., histone acetylation). DNA methylation is a process where a methyl group is covalently linked to cytosine [88]. Methylation patterns are established during early embryonic development and maintained by DNA methyltransferases [84]. In general, DNA methylation in regulatory regions (e.g., promoters) reduces gene expression and represents an important mechanism for tissue-specific gene silencing [89]. In contrast, demethylation leads to gene activation [88]. When methylation occurs in a gene promoter, the methyl group can interfere with transcription factor binding. Alternatively, gene silencing may occur through targeting of DNA-binding proteins. MeCP-1 and MeCP-2 (methyl cytosine binding proteins 1 and 2) recognize methylated DNA and recruit corepressors and histone modifying enzymes such as histone deacetylases and histone methyltransferases to methylated genes, precipitating an inactive chromatin configuration [88]. Important to this proposal, key genes that regulate HPA function (GR, CRH, POMC, and 11β-HSD-2) have been shown to be epigenetically regulated [82, 89–93]. Altered gene promoter methylation has been identified in human diseases [90]. More recently, altered hippocampal GR promoter methylation patterns has been demonstrated in human suicide subjects [94]. Maternal stress/anxiety and dietary protein restriction during pregnancy [92, 93, 95–98] and altered levels of maternal care [82, 85, 99] can leave permanent epigenetic marks in the genome and result in stable life-long changes in gene expression in offspring. Szyf and Meaney have shown that increased maternal care in rats leads to demethylation and increased histone acetylation of the hippocampal *GR* promoter, which is maintained throughout life [82]. Importantly, this effect is gene-promoter specific; other nonhippocampal *GR* promoters are not affected. Demethylation was specific to the NGFI-A binding site in the *GR* promoter, resulting in increased NGFI-A binding and increased *GR* expression. This leads to increased glucocorticoid feedback and a decrease in HPA activity [82]. The reduced promoter methylation could be reversed in adulthood by central infusion of L-methionine (a methyl donor) [86]. In other studies, protein restriction during rat pregnancy has been shown to cause reduced methylation of specific hepatic gene promoters, including the *GR,* and a resultant increase in gene transcription in F_1 offspring. Again, this demethylation is gene-specific and can be prevented by folate (a methyl donor) supplementation during pregnancy [97]. Very recent rat and human studies have shown that maternal stress/anxiety during pregnancy leads to altered methylation of the hypothalamic *CRH* promoter (rat offspring) and *GR* promoter in umbilical cord blood mononuclear cells (humans) [92, 93]. However, of most importance, glucocorticoids have been shown to cause permanent demethylation of specific fetal hepatic gene promoters in late gestation [100]. This demethylation results in enhanced transcription factor binding, and this is maintained following glucocorticoid withdrawal indicating stability of the effect [100]. One route by which sGC may

modulate methylation status is through a reduction in folate availability. In this regard, Cushing's patients (with elevated plasma cortisol) exhibit hyperhomocysteinemia [101]. Hyperhomocysteinemia inhibits the activity of DNA methyltransferases and induces hypomethylation [102]. There is extremely strong evidence emerging indicating that fetal GC exposure, be it endogenous or exogenous in the form of ACS, has profound influences on the fetal epigenome.

8.2. Transgenerational Effects. Recent studies have begun to show that the effect of ACS exposure may not be restricted to the immediate offspring of the pregnancy at risk but may affect subsequent generations; this is known as "transgenerational effects". These effects have been observed in the rat model where the offspring of an F_1 progeny whose mothers were treated with dexamethasone in the final week of pregnancy were mated with males from the same prenatal treatment. Both the F_1 and F_2 generation offspring exhibited decreased weight at birth compared to controls and also demonstrated abnormal endocrine responses to glucose challenges [103]. The programming effects were transmitted by either maternal or paternal lines implying an epigenetic mechanism. Further studies are needed to elucidate these mechanisms and how this will translate into the clinical realm.

9. The Need for Focused Research on Long-Term Effects of ACS

Pregnant women at risk of preterm birth continue to be a major clinical obstetrical issue. A single course of ACS remains the standard of care in this clinical setting to optimize fetal lung maturity. Because of concerns of fetal growth alterations and potential neurodevelopmental impairments, systematic repetitive administration of ACS has not been adopted as standard care. However, because of the short-term benefits of reducing neonatal morbidity, the more recent Cochrane Review supports the use of this approach in identified high-risk women [42]. In addition, the concept of "rescue" ACS has emerged in which ACS is given again only when delivery has again become likely [104]. Recent data from two randomized trials demonstrate benefits in reducing acute neonatal pulmonary morbidity without noticeable effects on fetal growth [105, 106].

The long-term effects of antenatal sGC exposure on HPA axis activity and neurological function is well documented in animal studies, and it appears that sGC, regardless of dose exposure, is capable of affecting fetal HPA axis development causing permanent changes in the HPA axis that persists through life and is manifested by chronic illness and behavioral changes. The previous long-term studies of ACS have focused only on major neurodevelopmental difficulties (cerebral palsy, blindness, deafness, and cognition) to the age of 2 years. However, the more recent animal studies suggest that the long-term effects of any exposure to sGC are more related to behaviour and the cardiometabolic factors contributing to chronic mental and physical illnesses, none of which have been adequately assessed in the majority of the long-term human follow-up studies. In retrospective studies, there is some suggestion of the behaviour changes seen with repeated exposure to sGC [46]. If early exposure affects hippocampal structure and alters function as the myriad of studies in animal models suggest, then there is likely to be an explosion of cognitive challenges, behavior disorders, and, perhaps, the risk of psychological disorders [107]. Similarly, if there is significant alteration in the HPA axis, then a generation of children and perhaps beyond are prone to develop the chronic illnesses of adulthood, such as heart disease, hypertension, type 2 diabetes.

The use of ACS, single or multiple courses, in the obstetric management of women at risk for preterm birth is likely not to diminish. Further studies are required on how to optimize the use of ACS in those women who remain undelivered 7 to 10 days after receiving an initial course of ACS. In addition, it is critical to determine ACS role, regardless of dosage, in "fetal programming" and its potential impact on a generation of children as it relates to behaviour, learning skills, and the potential for chronic illness. If the impact can be identified, appropriate measures can be implemented to minimize its effect.

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Research Article

Relation of Growth Rate from Birth to Three Months and Four to Six Months to Body Mass Index at Ages Four to Six Years

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Background. While rapid early weight gain are common in children who become obese later in life, so is growth faltering in the first 3 months of life. *Objective*. We seek to determine what relationship weight gain in the first six months of age, separated into two 3-month periods, have with the BMI of children ages 4 to 6 years in an inner-city community. *Subjects*. A convenience sample cohort of 154 children attending an inner-city clinic. *Methods*. Consecutive charts were reviewed retrospectively. Age, gender, birth weight and weight change in the first and second 3 months of life were introduced as fixed factors using mixed linear models with BMI in years 4 to 6 as the dependent variable. *Results*. Weight change quartile in the first 3 months of life did not predict of BMI in years 4 to 6; however, weight changes quartiles during months 4 to 6 were significant predictors for subsequent overweight. *Conclusion*. The data presented herein suggest that, for this specific population, weight gain can be promoted when it is most essential. It is necessary, however, to identify intermediary variables that could affect outcomes in this and other communities.

1. Introduction

Growth failure in utero increases risk for both neurodevelopmental delays [1] and the elements of the Metabolic Syndrome (insulin resistance, coronary heart disease and hypertension) later in life [2, 3]. Paradoxically, continued growth failure limits impact of prenatal undernutrition on subsequent Metabolic Syndrome [4–6], but growth failure adds to the neurodevelopmental burden [7–9]. Thus absence of a prenatal: postnatal mismatch in nutrition diminishes risk for the health consequences and increases risk for developmental delay later in life in populations at risk for undernutrition [4–9].

There have been dramatic increases in prevalence of pediatric obesity in the United States over the last 30 years [10–12]. School age children in impoverished African and Hispanic ancestry families are just as likely to be "overweight" or "obese," defined as Body Mass Index (BMI) above the 85th percentile for age and gender, as they are to be lean [11, 13].

The objectives for the present study are to determine what relationship, if any, (1) birth weight and (2) weight gain in the first six months of life, divided as first 3 months and second 3 months, have with BMI of children at 4 to 6 years of age. Variables available for study include birth weight, weights of infants at return visits to clinic in the first 6 months of life, and measures of weight and height with calculated BMI at 4 to 6 years of age.

2. Subjects and Methods

The subjects were a convenience sample cohort of 154 children attending the Resident Continuity Clinic at Suite D in Brooklyn, New York. Consecutive charts were reviewed retrospectively for the 154 children who had presented themselves for the set of immunizations commonly received at four years of age. The children ranged in age from four to six years.

The Suite D clinic serves an inner-city population in which approximately 31% of the children live in families with incomes below the poverty level [14]. The racial distribution of Suite D families is 81% African ("black"), 10% Hispanic, 5% European ("white—not Hispanic"), 1.5% Asian, and 2.5% other classifications [15]; however, the racial

designations commonly used by the Census Bureau are not explanatory variables for this population, which is of predominantly Afro-Caribbean ancestry [16].

All children were born at the State University of New York-Downstate Medical Center. No charts were excluded. Multiple imputation of missing data was used to avoid low power and bias associated with complete set analyses [17]. SAS (SAS Institute, Cary, NC) PROC MI was used to impute 25 values from a normal distribution for each missing bodyweight value [18]. All data were obtained from patient records; no parental interviews were conducted.

Data points were added for all children over the subsequent 12 months, permitting assessment of BMI for children 4 to 6 years of age. Weight to the closest 10 grams and length at birth to 6 months of age and height at 4 to 6 years of age to the closest 0.5 cm were recorded with date of measurement using techniques described by Jelliffe [19].

Mixed linear models were constructed with BMI in years 4 to 6 as the dependent variable. Gender was introduced as a fixed factor as was age in years as use of a shorter age distribution would have increased the number of parameters in this complex model to the extent of unmanageability.

Following the postpartum age division made by Eid [20], birth weight and weight changes in the first 3 months and in the second 3 months of life were each divided into quartiles and added as factors. The presence of interactions among all of these factors was tested. A Handcock-Stein-Wallis structure was modeled for intrapatient covariance. Satterthwaite corrections to denominator degrees of freedom were applied. BMI scores were base-10 log-transformed to improve symmetry and homoscedasticity. Small numbers of outlying observations were excluded from analysis. Model-estimated BMI log-means with corresponding standard errors are reported. Software used was SAS Release 9.1.3 (SAS Institute, Cary, NC).

The Suite D clinic serves a predominately Afro-Caribbean population that is representative of the community at large. Families served receive broad access, which is made available through public programs including Medicaid and State Child Health Insurance. These programs require that incomes be below 2.6 times the United States poverty level of $22,500 per year for a family of 4 people [14]. Of note, children in families that do not have documentation of legal status in the United States are eligible for these child insurance programs [19].

Approval for the study was received from the Institutional Review Board of SUNY-Downstate Medical Center.

3. Results

Of the total of 699 observations of BMI during years 4 to 6 of life for the 154 patients, there were 366 observations belonging to 76 patients with weight recorded at both birth and 3 months; 280 observations belonging to 60 patients had weight recorded at birth, 3 and 6 months.

While girls were more likely to become overweight, gender was not a statistically significant predictor of BMI in years 4 to 6 of life ($F[1, 47] = 3.78, P = 0.058$). Neither birth weight quartile ($F[3, 47] = 1.62,\ P = 0.197$) nor weight gain quartile from birth to 3 months of age ($F[3, 47] = 0.26,\ P = 0.853$) was a significant predictor of BMI in years 4 to 6 of life. Weight change quartile during months 4 to 6 of life, however, was a significant predictor of subsequent BMI at 4 to 6 years of life ($F[3, 47] = 3.47,\ P = 0.023$).

Controlling for the other terms in the model, estimated log BMI (mean/standard error) in years 4 to 6 of life by weight gain category in months 4 to 6 is shown in Table 1. The means of the two lower quartiles differed significantly from those of the two upper quartiles. No statistically significant interactions among the predictors were detected.

The data presented herein show significant correlation between weight gain from four to six months of age and BMI at ages four to six years. These findings remained significant when adjusted for birth weight, age of measurement, and gender. By contrast, using the same data set, negligible and nonsignificant differences were found when comparing weight gain from birth to three months of age and BMI at ages four to six years; noneffect for weight gain measures below three months of age shown by F statistics were all less than 1.0.

4. Discussion

Prenatal experiences with nutritional deprivation have a profound impact on postnatal responses to abundance (Metabolic Syndrome) [1–6] or continued malnutrition (neurodevelopmental delay) [7–9]. Weight faltering, as described by Emond et al. in their review of the impact of infant growth on subsequent neurodevelopmental delay, is a common occurrence in this community [9].

The data presented herein provides evidence that weight gain in the first three months may not have the impact on subsequent overweight or obesity similar to that found for gain in the full first six months has. The data suggest that weight gain in the second three months following birth, however, is the significant predictor of Body Mass Index in years 4 to 6 in our population. The weight gain from birth to 3 months of age, however, did not contribute significantly to the prediction.

Findings for the full sample of 154 children, analyzed using techniques provided for disrupted series, remained robust when adjusted for birth weight, age of measurement, and gender (F test less than 1) [17].

Data from the present study showing weight gain for the first six months, considered in their entirety, are consistent with those of Eid [20], and of Dennison et al. [21] who observed that weight gain from birth to six months of life

> "...was associated with increased risk of being overweight at 4 years of age; these findings were independently of potential confounders" [21].

Our data for weight gain in the first three months are, however, inconsistent with those provided by Stettler et al. in a multicenter study of 19,497 infants with a wide variety of social and ethnic backgrounds [22]. These authors used a cut-off of 4 months of age finding that "[a] pattern of rapid

Table 1: Mean log BMI at 4 to 6 years of age as a function of weight gain in months 4 to 6 of life. The data show that there are significant differences between means for quartiles 1 and 2 and means for quartiles 3 and 4.

Quartile	Gain range from 4 to 6 months of age (kg)	Mean log BMI at 4 to 6 years of age	Standard error
1	<1.5	1.200	0.09
2	1.5 to 1.9	1.201	0.08
3	1.9 to 2.5	1.229	0.09
4	>2.5	1.232	0.08

weight gain during the first 4 months of life was associated with an increased risk of overweight status at age 7 years" [22]. A microanalysis of their data, taken from a subset of 300 African American children from inner-city Philadelphia, replicated the correlation of rapid gain to 4 months of age and subsequent finding of overweight [23].

Limitations of the present study include using BMI as an outcome, as this measure does not necessarily imply obesity [12, 22, 23]. While children with BMI level at or above the 85th percentile are said to be overweight or "at risk" for obesity [12], the distribution of fat and muscle accumulation varies by age, ethnicity, timing of the "Adiposity (or "BMI") rebound," birth weight, and rate of weight gain [12, 24, 25]. Thus, accurate measures of fat deposition rather than lean muscle mass cannot be determined from a retrospective analysis of weight and height measures [24]. While data provided by Hediger et al. [24] suggest that the principle component of excessive weight gain in childhood is body fat, Wells et al. [25] provide data suggesting that effects of birth weight and rate of weight gain may influence lean and fat components of BMI differently in differing populations.

An additional limitation is that our data are derived from measures of a population with the unique characteristics of central Brooklyn, NY, an inner-city borough of New York City with 2.3 million residents. There are 315,000 people in SUNY-Downstate's primary service area, the venue for the present study. The genetic, social, and economic influences on families attending Suite D are neither representative of the population of Brooklyn as a whole nor of other inner-city communities in the United States such as the one described by Stettler et al. [23]. This population is immigrant based from the Caribbean basin. The families live at or just above the poverty level which substantially influences parenting practices, food purchase and consumption patterns, and increases risk for obesity [12, 19, 26–30]. As Gopalan points out

> "Differences. . .. between communities can result in important differences with nutritional status (especially of children) between households, and between communities with nearly similar overall levels of dietary inadequacy" [30].

Other variables likely to affect propensity for weight gain would include both paternal and maternal obesity [12], surgery to correct obesity [31], parental education [32], and food insecurity in the family [33]. The patient records reviewed to develop the data set for the current study do not have data to show differences between the population surveyed and others.

Use of data from the present study of a special sample in other populations and circumstances would require an in-depth analyses of antecedents, behaviors, and outcomes. A larger population would permit a shorter distribution of ages for outcome measures; however, observations drawn from macrosocial studies should not deter investigation of specific populations with unique characteristics, nor should data accumulated in microstudies be applied generally without careful investigation of applicability.

5. Conclusion

The data presented herein suggest that, for this specific population, weight gain can be promoted when it is most essential. Experiences in different populations are likely to differ. All require careful investigation of the variables affecting weight gain in the first months of life.

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