Pregnancy Calcium-Deficiency, Offspring Insulin Resistance

Adverse nutritional conditions during pregnancy may permanently alter the structure or function of specific organs in the offspring, leading to various chronic diseases in adulthood. Maternal undernutrition, and the consequent low birth weight of offspring, predisposes the offspring to various diseases, including adult-onset insulin resistance syndrome. Calcium (Ca) plays an important role in the pathogenesis of insulin resistance syndrome. Cortisol, the most important glucocorticoid, is considered to lead to insulin resistance and metabolic syndrome. 11β-hydroxysteroid dehydrogenase-1 is a key enzyme that catalyzes the intracellular conversion of cortisone to physiologically active cortisol.

1. Calcium and Insulin Resistance

Ca is an important second messenger in signal transduction pathways that regulate a wide variety of processes, including gene expression, protein synthesis, secretion, muscle contraction, metabolism, and apoptosis [1]. A link between Ca intake and insulin resistance in obesity and metabolic syndrome has been identified in epidemiological studies [2][3]. Several observational prospective studies have also shown a relationship between low or insufficient oral Ca intake and the incidence of type 2 diabetes mellitus (DM2) [4][5] and metabolic syndrome [6]. It was shown that individuals who have poor Ca intake present higher body weight [7]. Furthermore, a Ca-rich diet is known to improve insulin sensitivity [8][9]. A systematic review of randomized clinical trials suggested that Ca supplementation induces a small, but statistically significant, weight loss in overweight and obese individuals [10]. Earlier dose-dependent meta-analyses of cohort studies have shown that dietary intake of Ca prevents the development of DM2 [11][12]. Recently Wu et al. reported that in the large prospective cohort study, higher serum Ca levels precede peripheral insulin resistance, and this relation plays a role in the development of hypertension [13].

However, the mechanisms underlying this relationship remain poorly understood. Dietary Ca appears to play a pivotal role in the regulation of energy metabolism and obesity risk. Ca has the ability to modulate energy metabolism through calciotropic hormone concentrations: calcitriol and parathyroid hormone (PTH) [7]. A high-Ca diet is known to attenuate body fat accumulation and weight gain during periods of overconsumption of an energy-dense diet and promote fat breakdown and preserve metabolism during periods of caloric restriction, thereby markedly accelerating the loss of weight and fat [14]. Thus, a diet that is poor in Ca could inhibit lipolysis, stimulate lipogenesis, and decrease lipid oxidation [15]. Severe Ca deficiency increases visceral fat accumulation, down-regulating genes associated with fat oxidation, and increases insulin resistance while elevating serum PTH in estrogen-deficient rats [16]. The specific roles of Ca signaling and endoplasmic reticulum stress affect the development of insulin resistance and atherosclerosis [17].

Vitamin D plays a major role in Ca ion homeostasis by regulating Ca transport and bone mineralization. Vitamin D deficiency is associated with direct effects on offspring health such as low birth weight, poor skeletal health, obesity, and insulin resistance [18][19]. Similarly, prenatal vitamin D deficiency is associated with increased insulin resistance and inflammatory mediators in childhood [20][21]. A study of rats revealed that offspring who were born from mothers under vitamin D deficiency had increased fatty acid and markers of inflammation and oxidative stress in the liver and higher prevalence of liver steatosis [22]. In a mouse study, maternal vitamin D deficiency induced structural remodeling of the pancreas and impaired insulin secretion due to reduced gene expression of PDX-1, which regulates the expression of GLUT2, glucokinase, and insulin in adult offspring [19]. Maternal Vitamin D deficiency in a large birth cohort involving Indian children predicted higher insulin resistance at 9.5 years old [18].

2. Calcium and Epigenetics

The effects of the nutritional status of the mother have been discussed for many years, and several studies have considered the nutritional status of the mother during pregnancy as an environmental epigenetic factor that may play an important role in fetal development [23]. Regulatory regions of the genome can be modified through epigenetic processes during prenatal life. The modification of chromatin and DNA contributes to a large well-documented process known as "programming". Programming of fetal insulin resistance was reported to be induced by intrauterine abnormal activation of inflammation, adipokines, and the endoplasmic reticulum stress [24]. The correlation between gut dysbiosis and metabolic disturbance has attracted attention. Li et al. reported that imbalance in maternal Ca intake promotes body weight gain in offspring, which may be mediated by calcium's modulation on the gut microbiota and lipid metabolism [25].

Epigenetics is the study of mitotically heritable alterations in gene expression potential that are not caused by changes in DNA sequences [26]. Epigenetic mechanisms, which are established during prenatal and early postnatal development, function throughout the lifetime of complex organisms to maintain the diverse gene expression patterns of different cell types. Several molecular mechanisms, including the methylation of cytosines within CpG dinucleotides, various modifications of the histone proteins that package DNA in the nucleus, and cell-autonomous expression of a myriad of auto-regulatory DNA-binding proteins, interact to perpetuate the regional chromatin conformation that dictates which genes will be transcriptionally competent in specific cell types [27]. Ca has been indirectly associated with epigenetic modifications [28]. Conjugated linoleic acid and Ca supplementation modified the methylation pattern of fatty-acid-related genes under a high-fat diet in adult mice [29].

3. 11β-Hydroxysteroid Dehydrogenase

3.1. Glucocorticoid and 11β-Hydroxysteroid Dehydrogenase-1

Preliminary data suggest that circulating cortisol concentrations are higher in patients with metabolic syndrome compared to healthy subjects [30][31][32]. Dysregulation of glucocorticoid action has been proposed to be one of the central features of metabolic syndrome [33]. In the major metabolic organs, tissue sensitivity and exposure to glucocorticoids are determined by the levels of intracellular peroxisome proliferator-activated receptor α (PPARα), glucocorticoid receptor (GR), and the activity of the microsomal enzyme 11β-hydroxysteroid dehydrogenase-1 (11β-HSD1). 11β-HSD1 converts inactive glucocorticoids (cortisone in humans and 11-dehydrocorticosterone in rodents) to their active forms (cortisol and corticosterone, respectively) [34]. 11β-HSD1 is highly expressed in liver and adipose tissue, where glucocorticoids reduce insulin sensitivity and action [34][35][36]. The activity of 11β-HSD1 in liver and adipose tissue might contribute to the development of several features of insulin resistance or metabolic syndrome [37][38][39]. Obese individuals have increased *11β-HSD1* mRNA in both subcutaneous and visceral fat tissue [40]. Experimental studies have shown higher *11β-HSD1* expression in adipose tissue associated with features of metabolic syndrome such as increased waist circumference and insulin resistance [41].

Harno et al. reported that liver-specific *11β-HSD1* knockout mice given low-dose 11-dehydrocorticosterone do not show any of the adverse metabolic effects seen in wild-type mice [42]. This result implies that liver-derived intra-tissue glucocorticoids, rather than circulating glucocorticoids, contribute to the development of metabolic syndrome and suggest that local action within hepatic tissue mediates these effects. In contrast, Morgan et al. reported that adipose-specific *11β-HSD1* knockout mice given higher dose glucocorticoids are protected from hepatic steatosis and circulating fatty acid excess, whereas liver-specific *11β-HSD1* knockout mice develop full metabolic syndrome phenotypes [43]. This result demonstrates that 11β-HSD1, particularly in adipose tissue, is key to the development of the adverse metabolic profile associated with circulating glucocorticoid excess.

11β-hydroxysteroid dehydrogenase-2 (11β-HSD2) converts excess cortisol into inactive cortisone [44]. Phosphoenolpyruvate carboxykinase (PEPCK), a key hepatic gluconeogenic enzyme, simultaneously decarboxylates and phosphorylates oxaloacetate into phosphoenolpyruvate in one of the earliest rate-limiting steps of gluconeogenesis. 11β-HSD1 regulates key hepatic gluconeogenic enzymes, including PEPCK, through the amplification of GR-mediated tissue glucocorticoid action [34][36][44].

3.2. A Calcium-Deficient Diet Affects Hepatic 11β-Hydroxysteroid Dehydrogenase-1 Expression in the Liver of Dams

We previously reported that after 2 weeks of the low-Ca diet (low-Ca group: 0.008% Ca) or control diet (control group: 0.90% Ca), no differences in serum glucose, corticosterone, or insulin levels were observed between the two groups. In adulthood, 1 rat month is comparable to 3 human years [30]. The homeostasis model assessment of insulin resistance

(HOMA-IR) has proved to be a robust tool for the assessment of insulin resistance [45]. The low-Ca group rats showed higher values of HOMA-IR ($p < 0.05$) and intact parathyroid hormone ($p < 0.05$) and lower values of adiponectin ($p < 0.01$). In the low-Ca group, the expression of hepatic *Hsd11b1* mRNA was up-regulated, and hepatic *Pck1* expression was down-regulated ($p < 0.001$). The expression levels of *Nr3c1*, *Ppara,* and *Hsd11b2* showed a similar tendency. The 2-week Ca-deficient diet in rats was associated with the upregulation of the hepatic expression of *Hsd11b1* mRNA, which occurred before the animals developed obesity or overt features of metabolic syndrome [46]. Over-activity of 11β-HSD1 is associated with increased intracellular active glucocorticoids [34][35][36]. Rodent genetic studies have suggested that increased *Hsd11b1* expression or activity increases the risk of several components of metabolic syndrome [37][38]. In summary, a low-Ca diet alters glucocorticoid metabolism, which leads to hepatic upregulation of *Hsd11b1*, and is possibly a key mechanism of the induction of metabolic complications caused by Ca deficiency [46].

▌ 4. A Ca-Deficient Diet in Pregnant or Nursing Rats Affects the Offspring

Lillycrop et al. reported that pregnant rats on a protein-restricted diet developed hypomethylation and increased expression from the *Ppara* and *Nr3c1* promoters in the liver of the offspring [47][48]. This demonstrates that maternal nutrition during pregnancy can affect the regulation of non-imprinted genes via the altered epigenetic regulation of gene expression, thereby inducing different metabolic phenotypes. A high-fat diet during pregnancy was reported to induce neonatal gender-specific hepatic fat accumulation by increased *pck1* expression and histone modification [49].

4.1. The Methylation of Specific Cytosines within the 11β-Hydroxysteroid Dehydrogenase-1 Promoter in the Liver of the Offspring

We investigated the methylation of individual CpG dinucleotides in glucocorticoid-related genes in liver tissue of neonatal offspring from Ca-deficient rat dams. Female rats consumed either a Ca-deficient (0.008% Ca) or control (0.90% Ca) diet ad libitum from 3 weeks before conception to 21 days after parturition. Pups were allowed to nurse from their original mothers and were then sacrificed on day 21. The methylation of CpG dinucleotides in the *Pck1* [50], *Ppara, Nr3c1*, *Hsd11b1*, and *Hsd11b2* promoters was measured in liver tissue by pyrosequencing [42]. The methylation levels of all genes did not differ between groups, except for *Hsd11b1*, which was significantly lower in the rats from the Ca-deficient dams ($p < 0.05$). Serum corticosterone levels were higher in the male pups from the Ca-deficient dams than in those from the control dams ($p < 0.05$). The expression levels of *Pck1* and *Nr3c1* were significantly lower in the Ca-deficient group than in the control group, whereas those of *Hsd11b1, Hsd11b2,* and *Ppara* did not differ significantly [51].

Although the hepatic expression of *Hsd11b1* may have been initially up-regulated by epigenetic mechanisms in the offspring from Ca-deficient dams, *Hsd11b1* was likely down-regulated by other mechanisms during the early postnatal period. The methylation level of hepatic *Hsd11b1* was altered in the offspring as a consequence of the maternal dietary manipulation, but the epigenetic changes were not reflected in corresponding alterations in transcription. The nuclear receptor co-repressor complex is affected by environmental factors such as nutrients and hormones, which can lead to altered DNA methylation, acetylation, histone modification, other epigenetic changes, or some combination thereof; such epigenetic changes can and do alter the activity of DNA. These factors can also alter feedback loops involving nuclear receptors that normally regulate repression and maintain balance [52].

The down-regulation of *Hsd11b1* suggests that a compensatory mechanism may diminish cortisol production in the liver. Reduced hepatic glucocorticoid exposure also represents a compensatory mechanism that limits the metabolic complications of insulin resistance. In our study, no significant difference in serum 11β-HSD1 levels was found among the offspring groups; however, this may have been due to tissue-specific differences between serum and liver. Whether glucocorticoids modulate *Hsd11b1* expression is unknown, and *Hsd11b1* expression differs greatly between the liver and other tissues [53][54][55]. Obese rodents exhibit tissue-specific dysregulation of 11β-HSD1; it is usually up-regulated in adipose tissue and down-regulated in the liver [56][57]. In both obese Zucker rats and obese humans, 11β-HSD1 activity is high in adipose tissue but low in the liver [54][55][58]. In adipose tissue and smooth muscle cells, glucocorticoid induces *Hsd11b1* mRNA expression, but contradictory results have been obtained in the liver [55][58].

In summary, a Ca-deficient diet during pregnancy and nursing induced hypomethylation of specific CpG dinucleotides in the *Hsd11b1* promoter in the liver tissue of neonatal offspring. These changes in *Hsd11b1* expression likely contribute to marked increases in glucocorticoid hormone action in liver tissue [44] and potentiate the induction of insulin resistance during adult life [33].

4.2. A Ca-Deficient Diet in Dams during Gestation Increases Insulin Resistance in Male Offspring

The offspring rats of the same experimental methods as described in the previous section were raised to adults. Pups were allowed to nurse from their original mothers until weaning, when they were fed a control diet. The offspring were then sacrificed at an age of 180 days. The mean levels of insulin and glucose as well as the HOMA-IR values were higher only in the male offspring from the Ca-deficient dams than in those from the control dams ($p < 0.01$) [59]. In all offspring, the serum leptin levels were correlated with the serum insulin levels, and they were inversely correlated with the levels of ionized Ca.

A Ca-deficient diet in dams during gestation and early nursing may alter the glucocorticoid metabolism of her offspring, resulting in higher intracellular glucocorticoid concentration in the hepatic cells of the offspring; this abnormal glucocorticoid metabolism may induce the metabolic complications associated with Ca deficiency. Dietary Ca restriction in dams during pregnancy alters postnatal growth, the expression of *Hsd11b1*, and insulin resistance in a sex-specific manner.

4.3. Osteocalcin in the Offspring from a Ca-Deficient Dams

Osteocalcin (OC), or bone γ carboxyglutamic acid (Gla) protein, is the most abundant non-collagenous bone matrix protein [60]. OC is specifically expressed in osteoblast lineage cells and secreted from bone into the bloodstream [61]. OC is subjected to post-translational carboxylation by a vitamin K-dependent carboxylase to yield carboxylated (Gla-OC) and undercarboxylated (Glu-OC) molecules [62]. Glu-OC acts directly on pancreatic β-cells to increase insulin secretion, as well as insulin sensitivity and glucose tolerance [63][64][65]. The offspring rats of the same experimental methods as described in the previous section were raised to adults [59]. The mean levels of Glu-OC in Ca-deficient female offspring were higher than those in control female offspring and control male offspring. The mean levels of Gla-OC were higher in Ca-deficient female offspring than those in control female offspring, whereas no significant difference was observed in these measures between the two groups in male offspring. The effects of Glu-OC on glucose homeostasis have been reported to differ by sex [66]. Increased Glu-OC could contribute to lower insulin resistance in female Ca-deficient offspring, and therefore might be beneficial for glucose metabolism. Consequently, only male Ca-deficient offspring may acquire insulin resistance.

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