MUSCULOSKELETAL PATHOLOGY

Intrauterine Programming of Glucocorticoid—Insulin-Like Growth Factor-1 Axis—Mediated Developmental Origin of Osteoporosis Susceptibility in Female Offspring Rats with Prenatal Caffeine Exposure

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Epidemiologic investigations suggest that excessive intake of caffeine during pregnancy is one of the risk factors for osteoporosis in adult offspring. However, the phenomena and mechanisms have remained obscure. This study found that prenatal caffeine exposure (PCE) leads to persistent bone dysplasia in gestational day 20 and postnatal week 12 offspring rats and increases the susceptibility to osteoporosis in postnatal week 28 offspring rats. In the embryonic period, PCE increases the concentration of serum corticosterone and inhibits the expression of insulin-like growth factor-1 (*IGF1*) and osteogenic differentiation genes. After birth, the recovery of *IGF1* expression in PCE offspring is unable to completely compensate osteogenic function, and chronic stress can lead to a further decrease in *IGF1* expression. *In vitro* experiments found that corticosterone instead of caffeine restrains mineralized nodule formation and osteoblast differentiation by inhibiting *IGF1* expression. The corticosterone inhibits H3K9 and H3K14 histone acetylation of *IGF1* in osteoblasts through glucocorticoid receptor and CCAAT and enhancer binding protein α , respectively. In conclusion, glucocorticoid instead of caffeine inhibits bone *IGF1* expression via glucocorticoid receptor and CCAAT and enhancer binding protein α and mediates the PCE-induced bone dysplasia and bone mass reduction in offspring fetal rats, which may contribute to osteoporosis susceptibility in adulthood. (*Am J Pathol* 2018, 188: 2863–2876; <https://doi.org/10.1016/j.ajpath.2018.08.008>)

Osteoporosis is a common bone metabolic disease characterized by bone mass reduction and biomechanical weakening.^{1,2} The cause of primary osteoporosis remains unclear, hampering the early prevention and treatment of this disease. It is known that adverse prenatal environments can result in fetal dysplasia characterized by intrauterine growth restriction (IUGR) and increasing susceptibility to a variety of diseases in adulthood.^{3–5} Epidemiologic investigation shows that low birth weight is closely related to the loss of bone mass in adults,⁶ suggesting that part of osteoporosis originated from intrauterine dysplasia. However, the

mechanism of fetal-origin adult osteoporosis has not yet been reported.

Exorbitant concentrations of plasma glucocorticoids during the intrauterine period are reported to play an important role in the programming of fetal-origin adult diseases.^{7,8} Through the regulation of a series of neuroendocrine metabolic processes,

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glucocorticoids can cause sustained structural and functional changes in fetal tissue. Therefore, the downstream target genes regulated by glucocorticoids are of great interest. Insulin-like growth factor-1 (IGF1) plays an important role in the concentration and differentiation of stem cells (including embryonic stem cells and mesenchymal stem cells), thereby influencing the structural and functional development of various organs during fetal development.^{9,10} IGF1 can promote the proliferation and differentiation of osteoblasts through its downstream pathways in the processes of bone reconstruction and the maintenance of peak bone mass.¹¹ Although high concentrations of glucocorticoids can inhibit IGF1 expression in multiple tissues or cells,^{12,13} the molecular mechanism of this process has not been clearly elucidated. The glucocorticoids can suppress IGF1 expression by activating the glucocorticoid receptor (GR),¹⁴ and activation of the GR can result in developmental problems in multiple fetal tissues and cells.^{15,16} The CCAAT/enhancer binding protein α (C/EBP α) is one of the most important transcription factors that can collaborate with GR in regulating downstream gene expression.¹⁷ Thus, high concentrations of glucocorticoids may regulate IGF1 expression and the differentiation of osteoblasts through GR and C/EBP α .

Caffeine is one of the most frequently consumed pharmacologically active substances and is present in coffee, tea, soft drinks, food, and various drugs.¹⁸ Clinical investigations and animal experiments have found that prenatal caffeine exposure (PCE) can exert toxic effects toward the reproductive system and embryo, such as abortion and IUGR.^{19,20} Our previous studies found that PCE causes fetal overexposure to maternal glucocorticoids through the down-regulation of placental 11 β -hydroxysteroid dehydrogenase.^{21–25} In addition, PCE leads to significant increases in fetal serum corticosterone levels, whereas the IGF1 levels in serum and multiple tissues (eg, liver, skeletal muscle, cartilage, and adrenal gland) are notably decreased, accompanied by corresponding organ dysplasia and dysfunction in fetal rats.^{21–25}

The current study addresses whether PCE can lead to bone mass reduction during skeleton development and enhance osteoporosis susceptibility in adulthood. It further explores the intrauterine programming mechanism.

Materials and Methods

Materials

Caffeine (CAS number 58-08-2, >99% purity) was purchased from Sigma-Aldrich Co., Ltd. (St. Louis, MO). IGF1 (catalog number RP-8604) was purchased from Thermo Fisher Scientific Co., Ltd. (Walkersville, MD). Pentobarbital sodium (lot number 69020181) was obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Rat corticosterone enzyme-linked immunosorbent assay kits were obtained from Sangon Biotech Co., Ltd. (Shanghai, China). Primary antibody anti-IGF1 (sc9013) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The GR and C/EBP α

siRNA oligos were purchased from Gene Pharma Co. (Jiangsu, China). Real-time quantitative RT-PCR (RT-qPCR) kits were purchased from Takara Biotechnology Co., Ltd. (Dalian, China). The oligonucleotide primers for rat RT-qPCR genes (polyacrylamide gel electrophoresis purification) were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China). Dulbecco's modified Eagle's medium/F12 (1:1) and fetal bovine serum were supplied by Gibco (St. Louis, MO). Other chemicals and agents were of analytical grade.

Animals and Treatment

Specific pathogen-free Wistar rats (number 2012-2014, certification number 42000600002258, license number SCXK) weighing a means $\pm$ SD of 209 $\pm$ 12 g (females) and 258 $\pm$ 17 g (males) were obtained from the Experimental Center of the Hubei Medical Scientific Academy (Wuhan, China). Animal experiments were performed in the Center for Animal Experiment of Wuhan University (Wuhan, China), which is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. The Committee on the Ethics of Animal Experiments of the Wuhan University School of Medicine approved the protocol (permit number 14016). All animal experimental procedures were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals of the Chinese Animal Welfare Committee.

Animals were housed (room temperature, 18°C to 22°C; humidity, 40% to 60%), acclimated, and mated. Gestational day (GD) 0 was determined on confirmation of mating by the appearance of sperm in a vaginal smear. Pregnant rats were transferred to individual cages and then randomly divided into the control group and PCE groups. From GD9 to GD20, the low, PCE medium, and high PCE groups comprised the rats intragastrically administered caffeine at doses of 30, 60, and 120 mg/kg per day, respectively, and the control group was administered the same volume of distilled water. On GD20, the pregnant rats were euthanized under diethyl ether anesthesia. To reduce the differences caused by different litter sizes, rats with litter sizes of eight to 14 pups were considered qualified, and eight rats from each litter were randomly used. Each fetal-placental unit was quickly removed from the uterus, and the fetuses were weighed after being dried on filter papers. IUGR was diagnosed when the body weight of a fetus was 2 SDs less than the mean body weight of fetuses in the control group.²² The fetuses from each littermate were decapitated to collect whole blood. Then, the whole blood of same-sex fetuses ($n = 4$ to 7) from each litter was merged, and serum was obtained by centrifugation for subsequent analyses. Fetal femurs were separated under a dissection microscope and frozen in liquid nitrogen followed by storage at -80°C . Portions of the fetal femurs ($n = 5$) that were obtained from different litters were fixed in 4% paraformaldehyde for morphologic observation.

A proportion of the pregnant rats in the control and high PCE groups were kept until normal delivery. The number of

pregnant rats with a litter size of eight to 14 pups at birth was assigned to eight 8 for each group. To ensure adequate and relatively equal intake of nutrients during the suckling period, the litter size was randomly reduced to eight pups per litter (four male pups and four female pups). From postnatal week (PW) 1, all the pups were weighed weekly until PW12, and the female pups in each litter were randomly divided into four batches according to the different stages of life, denoted by PW12 with or without chronic stress modeling and PW28 with or without ovariectomy modeling. In reference to the chronic stress modeling, one female pup per litter was randomly selected from each group at PW10, and each female was given a 5-minute water (5°C to 7°C) swim exercise daily to induce chronic stress until PW12. For the ovariectomy modeling, one female pup per litter was randomly selected from each group, and each female pup underwent a bilateral ovariectomy at PW22 to induce osteoporosis until PW28.²⁶ The sham operation group was established as the control for ovariectomized rats. Animals of the sham operation group performed the same procedure in the ovariectomized operation at PW22, but the ovaries were not removed, and then the animals were fed normally to PW28.

The corresponding batches of rats were euthanized under pentobarbital sodium (40 mg/kg) i.p. injection anesthesia and decapitated at the scheduled time to collect blood and spongy bone tissue samples. Serum samples and spongy bone tissue samples were frozen in liquid nitrogen, followed by storage at -80°C for subsequent analyses. Portions of the tibia samples were fixed in 4% paraformaldehyde for morphologic observation. Half of the femur samples at PW28 were stored at -80°C for microcomputed tomography and bone biomechanics tests, which are commonly used for osteoporosis evaluation.

Serum Corticosterone Concentration Analysis

The concentration of serum corticosterone was measured by an ELISA kit following the manufacturer's protocols. The intra-assay and inter-assay CVs for corticosterone determination were 5.0% and 7.2%, respectively.

von Kossa Staining

For von Kossa staining, sections were deparaffinized and stained with 5% AgNO₃ until they became dark brown. Bone histomorphometry parameters, including trabecular bone volume/tissue volume (BV/TV), trabecular number (Tb.N), trabecular width (Tb.Wi), and trabecular separation (Tb.Sp), were measured on von Kossa-stained sections according to a previous study.²⁷

Immunohistochemistry and Analysis

Immunohistochemistry procedures were performed using a streptavidin-peroxidase conjugated method according to the manufacturer's instruction. Paraffin-embedded tissues were

cut into 4-μm serial sections and stained with rabbit anti-IGF1 monoclonal antibody (1:100 dilutions). At least five random fields from each section were examined. The signal was visualized using light microscopy and imaged, positively stained areas were analyzed, and the optical density of IGF1 staining from each image was counted by the Photo Imaging System (H550S, Nikon, Tokyo, Japan).

Microcomputed Tomographic Analysis

Femur samples were harvested at PW12 and PW28, and the attached soft tissue was thoroughly removed and fixed in 70% ethanol. The fixed femur was analyzed using microcomputed tomography (vivaCT40) and IPL software version 5.15 (Scanco Medical AG, Brüttisellen, Switzerland). Scanning parameters for each sample were as follows: integration time, 200 milliseconds; voltage, 70 kVp; and intensity, 114 μA. The region of trabecular bone for analysis and reconstruction was drawn starting from the distal epiphyseal growth plate and extended proximally for a total of 10% of the femoral length. The BV/TV, Tb.N, Tb.Sp, trabecular thickness (Tb.Th), connectivity density (Conn.D), and structure model index (SMI) were determined.²⁸

Biomechanical Testing

The specimens were thawed overnight at room temperature, and then the femur was separated by resecting the attached soft tissue. Biomechanical testing was performed using a materials testing machine (805, Instron Co., Norwood, MA). The sample was then loaded to failure by three-point bending under a displacement rate of 5 mm/minute. Maximum load, elastic load, break load, and stiffness were determined from the displacement-load curve.

Primary Cell Culture and Treatment

Calvariae were harvested from newly born Wistar rats, minced, and digested with collagenase A (c-2674, Sigma-Aldrich, St. Louis, MO) to obtain primary preosteoblasts as previously described.^{29,30} Then, cells were plated into six-well plates at a density of 2×10^5 cells per well and cultivated with osteogenic medium (Dulbecco's modified Eagle's medium/F12 with 10% fetal bovine serum, 50 μg/mL of ascorbic acid, 10 mmol/L β-glycerophosphate, 100 mg/mL of streptomycin, and 100 U/mL of penicillin). After cell confluence reached approximately 90%, osteoblasts were treated with corticosterone (250, 500, or 1250 nmol/L) and caffeine (1, 10, or 100 μmol/L) for 48 hours or 7 days and then harvested for gene expression analysis and von Kossa staining, respectively. To further confirm that corticosterone inhibited the differentiation of osteoblasts via IGF1, the osteoblasts were incubated with corticosterone (1250 nmol/L) in the absence or presence of 100 ng/mL of exogenous IGF1 (RP-8604, Thermo Fisher Scientific) for 7 days. To further confirm the role of GR and C/EBPα in gene regulation of corticosterone, the osteoblasts

were incubated with corticosterone (1250 nmol/L) in the absence or presence of GR and C/EBP α siRNA for 48 hours.

siRNA Knockdown

The osteoblasts were seeded into six-well plates (2×10^5 per well), and cell confluence was approximately at 70% for transfection. Using Dulbecco's modified Eagle's medium/F12 serum-free medium (250 μ L) to dilute siRNA oligo (40 pmol) and lipofectamine reagent (10 μ L), respectively, the two kinds of diluents were mixed to prepare the siRNA interfering reagents. The interfering reagents were evenly dripped into six-well plates (500 μ L per well), and cells were incubated at 37°C for 8 to 12 hours to complete the transfection.

Chromatin Immunoprecipitation Assay for Histone Acetylation of *IGF1*

Primary ossification centers (POCs; 50 mg) and cancellous bone samples (50 mg) were taken from the femurs of GD20 and PW12 offspring rats, respectively, and homogenized into cell suspension. The cell sediments were sheared to DNA fragments with lengths of approximately 200 to 800 bp with an ultrasonic pulverizer (VCX 130PB, Sonics & Materials Inc., Newtown, CT) under the addition of ultrasonographic pyrolysis liquid (0.5% SDS, 50 mmol/L Tris hydrochloride, and 5 mmol/L EDTA). Chromatin was incubated overnight at 4°C on a nutator/rocker to combine with antibody in the presence of 800 μ L of chromatin immunoprecipitation dilution buffer (20 mmol/L Tris hydrochloride, pH 8.0, 150 mmol/L sodium chloride, 2 mmol/L EDTA, and 1% Triton X-100), 1 μ L of antibody (eg, catalog number A7254, A7255, ABclonal, Woburn, MA), and 30 μ L Tris-EDTA buffer-bovine serum albumin-treated beads (product code 17061801, GE Health Care, Chicago, IL). The immunoprecipitated DNA-protein complex that was linked to the beads was collected by centrifugation. The DNA-protein complex was eluted with

elution buffer (1% SDS, 0.1 mol/L NaHCO $_3$) that contained protein K in a 65°C water bath overnight. These samples were subsequently purified using DNA purification kits, and then the isolated DNA was assayed using RT-qPCR.

Total RNA Extract and RT-qPCR

Total RNA of fetal POCs, PW12 spongy bone of tibia and osteoblasts was isolated using TRIzol reagent following the manufacturer's protocol. The total RNA was reverse transcribed using a first-strand cDNA synthesis kit. The cDNA was amplified using a one-step RT-qPCR reaction. The rat primer sequences and annealing temperature are given in the Table 1.

Statistical Analysis

SPSS software version 17 (SPSS Inc., Chicago, IL) was used for data analysis. Two-way analysis of variance was used to perform the multiple comparisons, such as for groups treated with PCE and ovariectomy as well as those treated with PCE and chronic stress. One-way analysis of variance followed by a Bonferroni post hoc test was used to perform multiple comparisons, such as different doses of drug treatment, in the *in vitro* experiments. A two-tailed *t*-test was performed on one factor of PCE. Statistical significance was designated as *P* < 0.05.

Results

Bone Mass and Biomechanics of PW28 Female Offspring Rats

To determine the increased susceptibility to osteoporosis in adult female offspring rats of the high PCE group (120 mg/kg per day, GD9 to GD20), ovariectomy procedures were performed at PW22 to induce osteoporosis, and then bone mass and biomechanical characteristics of femurs were measured at PW28 (Figure 1A). The femur bone mass of

Table 1 Primers Used for Real-Time Quantitative RT-PCR

Gene	Forward primer	Reverse primer	Product (bp)	Annealing temperature (°C)
<i>GAPDH</i>	5'-GCAAGTTCAACGGCACAG-3'	5'-GCCAGTAGACTCCACGACA-3'	156	60
<i>GR</i>	5'-CACCCATGACCCTGTCTAGTC-3'	5'-AAAGCCTCCCTCTGCTAACC-3'	151	60
<i>CEBPA</i>	5'-CCCCGAGTCACATCCCTATC-3'	5'-TCATAACTCCGCCATTCAC-3'	268	60
<i>IGF1</i>	5'-CTGGTGGAGCAGCAAGAGC-3'	5'-GTGGACAGTAGACGGAGGAAAG-3'	81	60
<i>IGF1R</i>	5'-GATGTCCCCTGCAATTACCA-3'	5'-TCTGTGCAAGTGATTTCGAGG-3'	108	58
<i>IRS1</i>	5'-CTGCAAGTTCAACGGCACAG-3'	5'-GTGCCAGTAGACTCCACGACA-3'	140	60
<i>AKT1</i>	5'-GCAAGTTCAACGGCACAG-3'	5'-GCCAGTAGACTCCACGACA-3'	155	60
<i>RUNX2</i>	5'-GAGCGTTCAACGGCACAG-3'	5'-GACAGTAGACTCCACGACA-3'	112	60
<i>SP7/osterix</i>	5'-GTGGATTCAACGGCACAG-3'	5'-GCCAGTAGACTCCACGACA-3'	123	60
<i>BGLAP/osteocalcin</i>	5'-CTGCGTTCAACGGCACAG-3'	5'-GTGGGTAGACTCCACGACA-3'	153	60
<i>COL1</i>	5'-TGTCGTTCAACGGCACAG-3'	5'-TGTTGGTAGACTCCACGACA-3'	148	60

AKT1, AKT serine/threonine kinase 1; *CEBPA*, CCAAT/enhancer binding protein α ; *COL1*, collagen type I; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; *GR*, glucocorticoid receptor; *IGF1*, insulin-like growth factor-1; *IGF1R*, insulin-like growth factor-1 receptor; *IRS1*, insulin receptor substrate-1; *RUNX2*, runt-related transcription factor 2.

the high PCE group was lower than that of the control group in nonovariectomized offspring rats, as characterized by decreases in BV/TV, Tb.N, and Tb.Th (Figure 1, B–D). This phenomenon was strengthened after ovariectomy, as characterized by the reduction of BV/TV, Tb.N, Tb.Th, and Conn.D (Figure 1, B–D and F) and increase of Tb.Sp and SMI (Figure 1, E and G). No differences were found in bone fragility between the PCE and control groups in nonovariectomized offspring rats. In ovariectomized offspring rats, the bone fragility was higher in the PCE group than in the control group, as characterized by decreased maximum load, elastic load break load, and stiffness (Figure 1, H–K). These results suggest that PCE can increase the susceptibility to osteoporosis in adult female offspring rats.

Long Bone Development and Related Gene (GR, C/EBP α , and IGF1 Pathway) Expression in GD20 Female Fetal Rats

To determine whether PCE-induced osteoporosis susceptibility has an intrauterine origin, the long bone

morphologic features and the changes of osteogenic differentiation–related gene expression were studied in female fetal rats. The femur lengths and POC lengths of the high PCE group were shorter than those of the control group (Figure 2, A and B). In addition, through quantitative analysis of mineralized area in POCs, the bone mass of the high PCE group was found to be lower than that of the control group, as characterized by decreased BV/TV, Tb.N, and Tb.Wi and increased Tb.Sp (Figure 2, C–F). Furthermore, the mRNA expression of osteogenic differentiation–related genes was tested by RT-qPCR, and the results revealed that the expression levels of runt-related transcription factor 2 (*RUNX2*), Osterix (*Osx*), collagen type I (*COL1*), and osteocalcin in the low, medium, and high PCE groups (30, 60, and 120 mg/kg per day, respectively, for GD9 to DG20) were significantly lower than those of their respective controls (Figure 2, G–J), indicating the presence of POC dysplasia and osteogenic differentiation disorder in PCE fetal rats.

To explore the mechanism of the POC dysplasia occurrence, the serum corticosterone concentrations and the expression of GR, C/EBP α , and IGF1 signaling pathway–related genes in POC were tested. The results

Figure 1 Effects of prenatal caffeine exposure (PCE) on bone mass and biomechanics of femur in offspring rats at postnatal week (PW) 28. **A:** Micro-computed tomogram. **B:** Bone volume/tissue volume (BV/TV). **C:** Trabecular number (Tb.N). **D:** Trabecular thickness (Tb.Th). **E:** Trabecular separation (Tb.Sp). **F:** Connectivity density (Conn.D). **G:** Structure model index (SMI). **H:** Femur maximum load. **I:** Femur elastic load. **J:** Femur break load. **K:** Femur stiffness. Data are expressed as means $\pm$ SEM. $n = 5$. * $P < 0.05$, ** $P < 0.01$ versus control. N, Newton; OVX, ovariectomized.

Figure 2 Effects of prenatal caffeine exposure (PCE) on bone morphologic features and mRNA expression of osteogenic differentiation-related genes in fetal rats on gestational day (GD) 20. **A:** Hematoxylin and eosin staining and von Kossa staining (red lines indicate ossification center length; red boxed areas, ossification areas). **B:** Primary ossification center (POC) length. **C:** Bone volume/tissue volume (BV/TV). **D:** Trabecular number (Tb.N). **E:** Trabecular width (Tb.Wi). **F:** Trabecular separation (Tb.Sp). **G:** Runt-related transcription factor 2 (RUNX2) mRNA expression in POC. **H:** Osterix mRNA expression in POC. **I:** Collagen type I (COL1) mRNA expression in POC. **J:** Osteocalcin mRNA expression in POC. Data are expressed as means $\pm$ SEM. $n = 5$ to 8 . $*P < 0.05$, $**P < 0.01$ versus control. Scale bar = $100 \mu\text{m}$.

indicate that serum corticosterone concentrations in the low, medium, and high PCE groups were higher than those in the control group (Figure 3A). The mRNA expression levels of GR and C/EBP α in the medium and high PCE groups were significantly higher than those in the control group (Figure 3, B and C). The mRNA expression levels of IGF1, insulin-like growth factor-1 receptor (IGF1R), insulin receptor substrate-1 (IRS1), and AKT serine/threonine kinase 1 (AKT1) in the low, medium, and high PCE groups were lower than those in the control group (Figure 3, D–G). Immunohistochemistry results revealed that IGF1 protein expression was lower in the POC of the high PCE group than that in the control group (Figure 3H). Chromatin immunoprecipitation experiments revealed that *IGF1* gene

(promoter region) H3K9 and H3K14 histone acetylation levels were lower in the POC of the high PCE group than those in the control group (Figure 3, I and J). These results suggested that PCE activates the GR and C/EBP α in POCs of fetal rats, with corresponding decreases in *IGF1* gene (promoter region) histone acetylation and IGF1 signaling pathway expression.

Long Bone Development and Related Gene (GR, C/EBP α , and IGF1 Pathway) Expression in PW12 Female Offspring Rats

To determine whether the above phenomenon in PCE fetal rats continued into postnatal stages, long bone

GD20

Figure 3 Effects of prenatal caffeine exposure (PCE) on mRNA expression of bone glucocorticoid receptor (GR), CCAAT and enhancer binding protein α (C/EBP α), and insulin-like growth factor-1 (IGF1) signaling pathway-related genes in fetal rats. **A:** Serum corticosterone (CORT) concentration. **B:** GR mRNA expression in primary ossification center (POC). **C–E:** mRNA expression of C/EBP α (**C**), IGF1 (**D**), and IGF1 receptor (IGF1R; **E**). **F:** Insulin receptor substrate-1 (IRS1) expression in POC. **G:** AKT serine/threonine kinase 1 (AKT1) expression in POC. **H:** IGF1 immunohistochemical staining and analysis (arrowheads indicate positive staining). **I:** Enrichment of H3K9 histone acetylation in IGF1 promoter. **J:** Enrichment of H3K14 histone acetylation in IGF1 promoter. Data are expressed as means $\pm$ SEM. $n = 5$ to 8. * $P < 0.05$, ** $P < 0.01$ versus control. Scale bar = 50 μ m. Original magnification: $\times 200$ (**H**). GD20, gestational day 20; MOD, mean value of optical density.

morphologic features and related gene expression were studied in PW12 female offspring rats. The femur length was not significantly different between the control and high PCE groups (Figure 4, A and C), but the femur bone mass of the high PCE group was lower than that of the control group, characterized by decreased BV/TV, Tb.N, Tb.Th, and Conn.D (Figure 4, D–G) as well as increased Tb.Sp and SMI (Figure 4, H and I). The mRNA expression of osteogenic differentiation-related genes was tested and *Osx*, *COL1*, and osteocalcin expression levels were significantly lower in the high PCE group than those in the control group (Figure 4B), indicating that the low bone mass of fetal rats in the high PCE group extended into adolescence.

To explore the potential mechanism responsible for bone mass loss in prenatal and postnatal PCE offspring rats, the serum corticosterone concentration and related genes (GR, C/EBP α , and IGF1 pathway) were examined in PW12 female offspring rats (including those with and without chronic stress for 2 weeks). In the offspring rats without

chronic stress, the serum corticosterone concentration was lower in the high PCE group than in controls (Figure 5A). In addition, the mRNA expression of GR and C/EBP α was lower than in the control groups (Figure 5, B and C). The mRNA expression of IGF1, IRS1, and AKT1 was higher than in the respective control groups (Figure 5, D and E), and the histone acetylation levels of H3K9 and H3K14 in the *IGF1* gene (promoter region) were also higher than those in the respective control groups (Figure 5, F and G). In the offspring rats with chronic stress, the serum corticosterone levels of the high PCE group were higher than those of the control group (Figure 5H). The mRNA expression of GR and C/EBP α was also higher than that in the control group (Figure 5, I and J). The protein and mRNA expression of IGF1 and mRNA expression of IRS1 and AKT1 were lower than those in the control group (Figure 5, K and L), and the histone acetylation levels of H3K9 and H3K14 in the *IGF1* gene (promoter region) were also lower than those in the control group (Figure 5, M and N).

Figure 4 Effects of prenatal caffeine exposure (PCE) on bone mass and mRNA expression of osteogenic differentiation-related genes in offspring rats at postnatal week (PW) 12. **A:** Femur morphologic features and length analysis. **B:** mRNA expression of osteogenesis differentiation-related genes. **C:** Micro-computed tomogram (micro-CT). **D:** Bone volume/tissue volume (BV/TV). **E:** Trabecular number (Tb.N). **F:** Trabecular thickness (Tb.Th). **G:** Connectivity density (Conn.D). **H:** Trabecular separation (Tb.Sp). **I:** Structure model index (SMI). Data are expressed as means $\pm$ SEM. $n = 5$ to 8. * $P < 0.05$, ** $P < 0.01$ versus control.

These results suggest that the recovery of bone IGF1 expression in PW12 PCE offspring rats is insufficient to compensate for intrauterine bone mass loss, and bone IGF1 exhibits low expression quickly after external stress. GR and CEBP α may mediate the regulation of bone IGF1 pathway expression by serum corticosterone.

Effects of Corticosterone on Osteoblast Differentiation and the Underlying Mechanism

To investigate whether corticosterone or caffeine inhibits the differentiation of osteoblasts and to identify the underlying mechanism, preosteoblasts were obtained from newly born rat skulls, placed in culture to promote osteogenic differentiation, and treated them with different concentrations (250, 500, 1250 nmol/L) of corticosterone and exogenous IGF1 (100 ng/mL) simultaneously and with different concentrations (1, 10, 100 μ mol/L) of caffeine alone for 7 days. The number of mineralized

nodules in the corticosterone (500, 1250 nmol/L)—treated wells was less than that in the control wells (Figure 6A). The number of mineralized nodules in the corticosterone (1250 nmol/L) and IGF1 (100 ng/mL)—cotreated wells was higher than that in the corticosterone (1250 nmol/L)—treated well. The mRNA expression of RUNX2, *Osx*, *COL1*, and osteocalcin was lower than those of the control in a concentration-dependent manner (Figure 6, B–E). The mRNA expression of RUNX2, *Osx*, *COL1*, and osteocalcin in the corticosterone (1250 nmol/L) and IGF1 (100 ng/mL)—cotreated wells was higher than that in corticosterone (1250 nmol/L)—treated wells. However, caffeine (1, 10, 100 μ mol/L) treatment had no effect on the formation of mineralization nodules (Figure 6F) or on RUNX2, *Osx*, *COL1*, and osteocalcin gene expression in osteoblasts (Figure 6, G–J). These results suggest that corticosterone (not caffeine) can inhibit osteoblast osteogenic differentiation and mineralization.

Figure 5 Effects of prenatal caffeine exposure (PCE) on mRNA expression of bone glucocorticoid receptor (GR), CCAAT/enhancer binding protein α (C/EBP α), and insulin-like growth factor-1 (IGF1) pathway-related genes in offspring rats on postnatal week (PW) 12. **A:** Serum corticosterone (CORT) concentration of PW12 offspring rats. mRNA expression of bone GR (**B**), C/EBP α (**C**), and IGF1 (**D**) pathway-related genes of PW12 offspring rats. **E:** IGF1 immunohistochemical staining and analysis of PW12 offspring rats. Enrichment of H3K9 (**F**) and H3K14 (**G**) histone acetylation in IGF1 promoter of PW12 offspring rats. **H:** Serum CORT concentration of PW12 offspring rats with chronic stress. mRNA expression of bone GR (**I**), C/EBP α (**J**), and IGF1 (**K**) pathway-related genes of PW12 offspring rats with chronic stress. **L:** IGF1 immunohistochemical staining and analysis of PW12 offspring rats with chronic stress. Enrichment of H3K9 (**M**) and H3K14 (**N**) histone acetylation in IGF1 promoter of PW12 offspring rats with chronic stress. Data are expressed as means $\pm$ SEM. $n = 5$ to 8. * $P < 0.05$, ** $P < 0.01$ versus control. Scale bar = 50 μ m. Original magnification: $\times 200$ (**E** and **L**). MOD, mean value of optical density.

To determine the mechanism of corticosterone inhibition of the osteogenic differentiation of osteoblasts, the GR, C/EBP α , and IGF1 pathway-related genes were measured after corticosterone treatment (250, 500, and

1250 nmol/L) for 48 hours. The mRNA expression levels of GR and C/EBP α were higher than those in the control group (Figure 7, A and B), whereas the mRNA expression of IGF1, IGF1R, IRS1, and AKT1 in the corticosterone

Figure 6 Effects of corticosterone treatment on osteogenic mineralization and osteogenic differentiation—related gene expression. **A:** von Kossa staining of osteoblasts treated with corticosterone and exogenous insulin-like growth factor-1 (IGF1) (arrowheads indicate mineralized nodules). mRNA expression of Runt-related transcription factor 2 (RUNX2) (**B**), osterix (**C**), osteocalcin (**D**), and collagen type I (COLI) (**E**) osteoblasts treated with corticosterone and exogenous IGF1. **F:** von Kossa staining of osteoblasts treated with caffeine (arrowheads indicate mineralized nodules). mRNA expression of RUNX2 (**G**), osterix (**H**), osteocalcin (**I**), and COLI (**J**) osteoblasts treated with caffeine. Data are expressed as means $\pm$ SEM. $n = 3$. * $P < 0.05$, ** $P < 0.01$ versus control; † $P < 0.05$, †† $P < 0.01$ versus corticosterone (1250 nmol/L). MOD, mean value of optical density.

(500, 1250 nmol/L) group were lower than those of the control group (Figure 7, C–F). Knockdown of the expression of GR or C/EBP α by siRNA partially reversed the inhibition of corticosterone on IGF1 mRNA and protein expression (Figure 7, G and H). Furthermore, knockdown of GR expression partially reversed the inhibition of corticosterone on H3K9ac (Figure 7I); however, knockdown of C/EBP α expression only partially reversed the inhibition of corticosterone on H3K14ac (Figure 7J). These results suggested that corticosterone regulates the expression of IGF1 in osteoblasts via GR and C/EBP α and participates in the regulation of corticosterone-mediated

H3K9 and H3K14 histone acetylation, respectively, in the promoter of the *IGF1* gene.

Discussions

PCE Induces Bone Mass Reduction and Enhances Osteoporosis Susceptibility in Adult Female Offspring

Numerous epidemiologic surveys have found that the bone mineral content and bone mineral density of femoral necks or lumbar spines in adults are positively related to birth weight,^{5,31}

Figure 7 Effects of corticosterone on mRNA expression of glucocorticoid receptor (GR), CCAAT/enhancer binding protein alpha (C/EBPα), and insulin-like growth factor-1 (IGF1) signaling pathway-related genes in osteoblasts. **A–E:** mRNA expression of GR (**A**), C/EBPα (**B**), IGF1 (**C**), insulin-like growth factor-1 receptor (IGF1R; **D**), and insulin receptor substrate-1 (IRS1; **E**). **F:** mRNA expression of AKT serine/threonine kinase 1 (AKT1). **G:** IGF1 mRNA expression after GR or C/EBPα siRNA knockdown. **H:** IGF1 protein expression determined by Western blot after GR or C/EBPα siRNA knockdown. Enrichment of H3K9 (**I**) and H3K14 (**J**) histone acetylation in IGF1 promoter after GR or C/EBPα knockdown by siRNA. Data are expressed as means ± SEM. $n = 3$. * $P < 0.05$, ** $P < 0.01$ versus control; † $P < 0.05$, †† $P < 0.01$ versus corticosterone (1250 nmol/L).

which indicates the existence of an intrauterine developmental origin of adult osteoporosis.³² Investigations found that the probability of osteoporotic hip fracture is higher in older women^{33,34} and suggest that osteoporosis is more typical in women and warrants further attention. Therefore, in the present study, female offspring rats were bred and the classic osteoporosis model (bilateral ovaries removed) established at PW22 according to previous reports.^{35,36} By testing the femur bone mass and biomechanical strength at PW28, it was confirmed that PCE can increase susceptibility to osteoporosis in female adult offspring (Figure 1).

Various adverse environments during pregnancy can lead to long bone dysplasia in offspring.^{37,38} Exposure to xenobiotics (eg, caffeine, nicotine, ethanol, and dexamethasone) during pregnancy can result in IUGR and long bone growth retardation in fetal rats.^{23,39–41} In the present study (Figures 2 and 4), PCE was found to lead to bone mass reduction from the prenatal to postnatal period, which was attributable to sustained poor differentiation of osteoblasts. Bone mass reduction is the main pathologic feature of osteoporosis. Bone development during the intrauterine period is associated with lower bone accrual in children and peak bone mass in adults.⁴² PCE-induced bone dysplasia and bone mass reduction may be one reason for the increased susceptibility to osteoporosis in adult offspring.

Intrauterine Programming of the Glucocorticoid-IGF1 Axis—Mediated PCE-Induced Bone Dysplasia and Bone Mass Reduction

Intrauterine programming is the adaptive response of the body to poor environments and serves as a mechanism of compensatory protection for the fetus, which might increase susceptibility to various diseases after birth.^{43,44} Moreover, high glucocorticoid levels during the intrauterine period can cause continuous changes in the structure and function of fetal tissues through regulation of a series of neuroendocrine metabolic processes.^{7,8} Our earlier studies suggested that programming of the glucocorticoid-IGF1 axis may influence PCE-induced multiorgan dysplasia.^{21,24}

In this study, it was confirmed that the glucocorticoid-IGF1 axis was involved in the bone dysplasia and bone mass reduction in the uterus (Figures 2 and 3). These axial changes of serum corticosterone concentration and bone IGF1 expression during the intrauterine period mediated by epigenetic modifications have a programming effect that can be extended to the postnatal period, resulting in a certain degree of recovery of bone development and bone mass (Figures 4 and 5). This intrauterine programming effect of the glucocorticoid-IGF1 axis was found to increase the sensitivity of the offspring to environmental factors (chronic stress) and resulted in bone

Figure 8 Intrauterine programming mechanisms of prenatal caffeine exposure—induced adult offspring osteoporosis susceptibility. The intrauterine period is shaded gray. C/EBP α , CCAAT/enhancer binding protein α ; GR, glucocorticoid receptor; IGF1, insulin-like growth factor-1.

mass reduction (Figure 5). It is significant that the PCE offspring rats had a more obvious bone mass reduction in the face of a clear inducement of osteoporosis (such as ovariectomy). Therefore, the intrauterine programming of the glucocorticoid-IGF1 axis is likely involved in bone dysplasia and bone mass reduction before and after birth.

Glucocorticoids Epigenetically Regulate Bone IGF1 Expression through GR and C/EBP α

Under physiologic conditions, C/EBPs can rapidly regulate downstream genes in concert with the glucocorticoid and GR,¹⁷ and C/EBPs are reportedly involved in the regulation of IGF1 and IGF1R expression in different tissues and cells.^{45,46} Maternal glucocorticoids regulate fetal adrenal IGF1 expression through GR and C/EBP α in offspring with prenatal caffeine or ethanol exposure.^{21,47} This study confirmed that PCE-induced high concentrations of maternal glucocorticoid can down-regulate IGF1 expression in bone tissues through up-regulating GR and C/EBP α (Figures 3 and 7).

Epigenetic modifications are sensitive to adverse environments during pregnancy⁴⁸ and are involved not only in organ development, intrauterine programming, and inter-generational genetic processes but also in the development of adult diseases of fetal origin, such as metabolic diseases. An increasing number of studies have suggested that maternal glucocorticoid overexposure attributable to adverse pregnancy environments is one of the main reasons for intrauterine programming.^{49,50} Intrauterine programming alterations caused by high glucocorticoid levels during pregnancy are likely to be associated with epigenetic modifications of important genes.^{7,8} Moreover, glucocorticoid overexposure during pregnancy can lead to changes in DNA

methylation in multiple organs of fetal rats.⁵¹ This study confirmed that maternal glucocorticoids down-regulate H3K9 and H3K14 histone acetylation of bone IGF1 through GR and C/EBP α , respectively (Figures 3 and 7).

The Rationale of the PCE-Induced IUGR Model and Its Advantages in the Study of Fetal-Originated Osteoporosis

Regarding caffeine doses and the IUGR model in the present study, epidemiologic evidence indicates that a caffeine intake of 300 mg/d (5 mg/kg per day) in pregnant women is associated with an increased risk of IUGR according to the World Health Organization.⁵² However, some studies have found that the caffeine intake of some pregnant women is >300 mg/d and may even reach 500 mg/d.⁵³ In previous studies,^{21,54} caffeine doses of 20, 60, and 180 mg/kg per day^{22,25} or 30, 60, and 120 mg/kg per day were used to induce IUGR, and abnormal expression was observed for multiple development-related genes (eg, adrenal steroidogenic acute regulatory protein and hepatic IGF1) in the low-dose caffeine group (ie, 20 or 30 mg/kg per day) with a positive dose-effect relationship. Using the dose conversion between humans and rats (human/rat 1:6.17 by body surface area comparisons),⁵⁵ caffeine at 20 or 30 mg/kg in rats is equivalent to 194 or 292 mg of caffeine per day for a 60-kg individual, which corresponds to one to two cups of coffee assuming that a standard cup of brewed coffee contains approximately 150 to 200 mg of caffeine. The doses in the high PCE group used in the present study represent a high caffeine intake by a pregnant human, which is probably not achieved in daily life. However, considering the stability of the IUGR model, a high dose of caffeine (120 mg/kg per day) was used to induce IUGR in rats, thus more fully exploring the potential molecular mechanisms of PCE-induced long bone dysplasia and osteoporosis susceptibility. Therefore, the results of this study suggested that the intake of caffeine during pregnancy may lead to an increased susceptibility to osteoporosis rather than direct occurrence of osteoporosis.

Conclusions

In summary, PCE can lead to bone dysplasia and bone mass reduction before and after birth and increase susceptibility to osteoporosis in adulthood. The underlying mechanisms are associated with osteogenic differentiation persistent disorder mediated by intrauterine programming of the glucocorticoid-IGF1 axis. The maternal glucocorticoid induced by PCE can inhibit the expression of the IGF1 pathway in fetal bone tissue by activating GR and recruiting C/EBP α , which leads to the osteogenic differentiation disorder and long bone dysplasia (Figure 8). Although the lengthwise catch-up growth of the long bone appeared after exiting the maternal environment, the osteogenic differentiation disorder caused by PCE remained. This resulted in lower bone mass and increased susceptibility to

osteoporosis after insults such as ovariectomy in adulthood. This study not only clarifies the molecular mechanism of PCE-induced long bone developmental toxicity but also provides a theoretical foundation for revealing maternal high glucocorticoid programming embryonic origin adult osteoporosis (Figure 8).

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