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The Maternal Diet during Pregnancy Programs Altered Expression of the Glucocorticoid Receptor and Type 2 11β-Hydroxysteroid Dehydrogenase: Potential Molecular Mechanisms Underlying the Programming of Hypertension *in Utero**

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ABSTRACT

Potential mechanisms underlying prenatal programming of hypertension in adult life were investigated using a rat model in which maternal protein intake was restricted to 9% *vs.* 18% casein (control) during pregnancy. Maternal low protein (MLP) offspring exhibit glucocorticoid-dependent raised systolic blood pressure throughout life (20–30 mm Hg above the control).

To determine the molecular mechanisms underlying the role of alterations in glucocorticoid hormone action in the prenatal programming of hypertension in MLP offspring, tissues were analyzed for expression of the glucocorticoid receptor (GR), mineralocorticoid receptor (MR), 11βHSD1, 11βHSD2, and corticosteroid-responsive Na/K-adenosine triphosphatase α1 and β1. GR protein (95 kDa) and messenger RNA (mRNA) expression in kidney, liver, lung, and brain was more than 2-fold greater in MLP *vs.* control offspring during fetal and neonatal life and was more than 3-fold higher during subsequent juvenile and adult life ($P < 0.01$). This was associated with increased levels of Na/K-adenosine triphosphatase α1- and β1-subunit mRNA expression. Levels of MR gene expression remained unchanged. Exposure to the MLP diet also resulted in markedly reduced levels of 11βHSD2 expression in the MLP placenta on days 14 and 20 of gestation ($P < 0.001$), underpinning similar effects on 11βHSD2 enzyme activity that we reported previously. Levels were also markedly reduced in the kidney and adrenal of MLP offspring during fetal and postnatal life ($P < 0.001$). This programmed decline in 11βHSD2 probably contributes to marked increases in glucocorticoid hormone action in these tissues and potentiates both GR- and MR-mediated induction of raised blood pressure. In contrast, levels of 11βHSD1 mRNA expression in offspring central and peripheral tissues remained unchanged.

In conclusion, we have demonstrated that mild protein restriction during pregnancy programs tissue-specific increases in glucocorticoid hormone action that are mediated by persistently elevated expression of GR and decreased expression of 11βHSD2 during adult life. As glucocorticoids are potent regulators not only of fetal growth but also of blood pressure, our data suggest important potential molecular mechanisms contributing to the prenatal programming of hypertension by maternal undernutrition in the rat. (*Endocrinology* **142:** 2841–2853, 2001)

HYPERTENSION, insulin resistance, type 2 diabetes, and other adverse risk factors for cardiovascular disease (CVD) are known to be associated with adult lifestyle factors such as smoking, excessive consumption of alcohol, saturated fat, physical inactivity, and obesity. However, there is an increasing body of robust epidemiological evidence from large cohorts of human populations worldwide indicating that the nutritional and hormonal environment encountered by the fetus is a strong determinant not only of fetal growth, but also of CVD risk in later life (1–4). Accordingly, individuals who at birth were of low body weight or who were thin in proportion to weight or short in proportion to head circumference have significantly greater risk of premature mortality from hypertension and CVD that is independent of adult lifestyle factors (1, 5). Maternal nutritional status during pregnancy is an important nongenetic determinant of fetal growth. Disproportionate diet composition (*e.g.* protein in relation to carbohydrate) during specific periods of pregnancy can result in persistent elevation of blood pressure in the offspring during later life (2, 3). This has led to the hypothesis that suboptimal maternal nutrition permanently modifies or programs fetal and adult morphology as well as metabolic and endocrine pathways, such that, through maladaptation to the postnatal environment, they confer greater risk of CVD in adult life (6).

These epidemiological data are strongly supported by experimental animal studies. In rodents both severe nutrient restriction to the fetus [through either uterine artery ligation (7) or major calorific restriction (8)] and mild undernutrition arising from a maternal low protein (MLP) diet during part or all of gestation result in offspring with low body weight and/or disproportionate body size at birth that have elevated blood pressure (8–10) and dysregulation of glucose metabolism in later life (11). Similarly, in the sheep, early to midgestation maternal nutrient restriction program raised blood pressure in the offspring (12).

The precise molecular mechanisms underlying the pro-

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gramming of adult disease by maternal undernutrition are unknown. However, recent experimental studies strongly suggest that fetal overexposure to maternal glucocorticoids (10, 13–16) triggers programming events *in utero* that establish persistent increases in glucocorticoid hormone action throughout life (14, 16). Glucocorticoids are potent regulators of fetal growth and development (17). They also promote increased blood pressure by potentiating tissue sensitivity to vasoactive hormones (18), promote gluconeogenesis (19), and antagonize the metabolic actions of insulin (20). Indeed, glucocorticoid excess results in a metabolic syndrome-like phenotype (21) similar to that programmed in human populations (22) and rat models (9–14). Programmed alterations in glucocorticoid action are likely, therefore, to play a key role linking intrauterine nutrient availability, fetal growth, and CVD risk.

Glucocorticoid hormone action within the cell is regulated by expression of the glucocorticoid receptor (GR) and isoforms of 11β-hydroxysteroid dehydrogenase (11βHSD1 and 11βHSD2) at the level of gene transcription (23, 24)., Most of the classical effects of glucocorticoid are therefore mediated by the GR (23), with levels of glucocorticoid binding largely dictated by levels of GR mRNA expression (23). In addition, 11βHSD1 behaves predominantly as an 11-oxo-reductase, catalyzing the conversion of cortisone to bioactive cortisol [11-dehydrocorticosterone (A) to corticosterone (B) in the rat], and acts as an intracellular amplifier of glucocorticoid access to the GR (23, 24). Conversely, 11βHSD2 behaves as an 11-dehydrogenase, catalyzing the inactivation of cortisol to cortisone (B to A in the rat). It maintains the aldosterone specificity of the mineralocorticoid receptor (MR) (24) and may also potentiate glucocorticoid hormone action through the GR (25).

11βHSD2 is also expressed at high levels in feto-placental tissues, where it is thought to protect the fetus from overexposure to glucocorticoid (13, 15, 24, 26, 27). In the rat the potent effects of the MLP diet on fetal growth and programming of hypertension and dysregulation of glucose metabolism are thought to be mediated by inhibition of placental 11βHSD2 activity (10, 13). In support of this hypothesis, maternal treatment during pregnancy with the 11βHSD inhibitor carbenoxolone mimics the effects of the MLP diet on offspring birth weight and later blood pressure (15). In another pharmacological model of programmed hypertension in which the pregnant dam is treated with dexamethasone, the offspring exhibit persistently elevated GR expression in the liver (16) and attenuated GR expression in the hippocampus (28). The molecular mechanisms underlying the intrauterine programming of hypertension by modest variations in the maternal diet during pregnancy (9), however, are unknown. In the present study we describe, for the first time, the prenatal programming effects of a maternal isocaloric low protein-high carbohydrate (MLP) diet on the expression of GR, MR, 11βHSD1, and 11βHSD2 and also the expression of corticosteroid-responsive Na/K-adenosine triphosphatase (Na/K-ATPase) α1- and β1-subunits (29) in feto-placental and postnatal central and peripheral tissues in the rat.

Materials and Methods

Materials

All reagents were of analytical or molecular biology grade and unless otherwise stated were obtained from Sigma-Aldrich Corp. (Poole, UK). Dietary components were obtained from Special Diet Services Ltd. (Cambridge, UK).

Animals

All animal experiments were performed in accordance with the provisions of Home Office Project Licenses PPL30/1522 and PPL30/1523 granted under the Animal Procedures Act 1986.

Virgin female Wistar rats (230–250 g; Harlan UK Ltd., Bicester, UK) were maintained under controlled lighting (lights on at 0700 h and off at 1900 h) and temperature (22 C) conditions and allowed *ad libitum* access to standard rat chow (56.3% carbohydrate, 18.3% protein, and 0.7% NaCl) and tap water for at least 1 week before the experimental protocol.

Experimental animal protocol

Virgin female Wistar rats were mated using one of four adult male Wistar rats (Harlan UK Ltd., Bicester, UK), with conception defined by the presence of a vaginal plug. Thereafter, the pregnant female rats were housed in pairs and randomly allocated to receive a control diet comprised of 18% casein or a low protein diet comprised of 9% casein as previously described (9) throughout pregnancy (term = 22 days). Pregnant rats were weighed at 4- to 5-day intervals and on day 18 were housed individually. A proportion of pregnancies (n = 10/diet group) were terminated on days 14 and 20 of gestation by euthanasia of the pregnant dam with barbiturate (100 mg/kg pentobarbital-sodium, *i.e.* Euthatal, PMB Animal Health, UK). Placentas and fetuses were excised and weighed. Fetal tissues were dissected and along with the placentas were snap-frozen in liquid nitrogen and stored at −80 C for molecular analyses. At term, offspring were also weighed within 6 h of delivery, sexed, and culled to eight (n = 4 of each sex) per litter. To further standardize the postnatal environment, the experimental diet was replaced with standard chow *ad libitum*. Culled offspring were dissected for excision of neonatal tissues, which were also snap-frozen and stored at −80 C.

After blood pressure measurements (described below), offspring were weighed, and two from each litter were randomly killed by CO_2 asphyxiation at 4, 8, 12, and 16 weeks of age. In additional litters offspring were randomly killed from each litter at 2, 6, 10, and 20 weeks of age. Organs were excised and stored at −80 C for molecular analyses. This protocol enabled relatively continuous analyses of offspring gene expression from birth to 5 months of age in which variation within each diet group at each time point reflected interlitter variability between pregnancies rather than intralitter variability from the same pregnancy.

Blood pressure measurements

Systolic blood pressure was determined by tail cuff plethysmography, which we described in detail previously (9). An IITC model 229 blood pressure recorder linked to a computer software package was used to determine blood pressure using a preset algorithm (Linton Instrumentation, Diss, UK). Tail cuffs were selected according to the size of each rat and were inflated to 300 mm Hg to occlude the tail arterial pulse. Deflation at approximately 3 mm Hg/sec allowed accurate determination of systolic pressure as the pulse returned. Each animal was acclimatized to this procedure before consecutive measurements (n = 4–5) on each rat over a period of less than 5 min between 1100–1300 h. The mean value was recorded. All blood pressure measurements were determined repetitively in the same animal to assess the reproducibility of recordings. A single operator blinded to the prenatal experience of the rats was employed in blood pressure measurements, resulting in intra- and interassay coefficients of variation of less than 5% and less than 8% respectively. This method has previously been validated against direct arterial cannulation measurements taken in conscious, unrestrained, animals from which a correlation coefficient of 0.974 was established between the two methods (30).

Molecular analyses

RNA and protein isolation from tissues. Total RNA and protein were isolated in parallel from the same tissue aliquot using Tri-Reagent (Sigma-Aldrich Corp., Poole, UK). Total RNA was also isolated using RNAzol B (Biogenesis, UK) as previously described (29, 31). Both procedures are modifications of the single step acidified phenol-chloroform extraction method. The integrity and quantification of total RNA, pooled from three separate aliquots of tissue from the same tissue sample, were assessed by comparison with RNA mol wt markers (Amersham Pharmacia Biotech-Pharmacia, Little Chalfont, UK) coelectrophoresed in an ethidium bromide-stained agarose gel and also by UV spectrophotometric absorbance at 260 nM. Protein was also isolated from the same sample by precipitation with propan-2-ol, centrifugation (10,000 × *g*, 20 min, 4 C), 90% ethanol wash, and resuspension in sterile Milli-Q water comprising 0.1% SDS and 1 mM phenylmethylsulfonylfluoride and was stored at −80 C. Protein concentrations were quantified by the Bradford method using a commercially available kit (Bio-Rad Laboratories, Inc., Hemel Hempstead, UK)

Northern blot analysis

Northern blot analyses were performed as previously described (25, 29). Briefly, total RNA (30 μg) was electrophoresed through an agarose (1.5%)/formaldehyde (15%)/3-[*N*-morpholino]propanesulfonic acid (MOPS) gel in MOPS buffer. RNA was transferred overnight onto Hybond N^+ nylon membrane (Amersham Pharmacia Biotech-Pharmacia) by capillary action facilitated by 20 × SSC (standard saline citrate) and then UV cross-linked (CL-1000 UV cross-linker, UVP, Wolf Laboratories, York, UK).

For complementary DNA (cDNA) probe hybridizations (11βHSD1, 11βHSD2, and Na/K-ATPase α1- and β1-subunits), each membrane was prehybridized at 65 C in hybridization buffer (0.77 M sodium phosphate, 5 mM EDTA, and 7% SDS, pH 7.2) containing 100 μg denatured salmon sperm (ss) DNA. Probe was added, and hybridization was performed in the same buffer for 16 h at 65 C. For complementary RNA (cRNA) probe hybridizations (GR and MR), membranes were prehybridized at 42 C in a rotary incubator (Hybaid, Ashford, MIDDX, UK) in hybridization buffer as described previously (25). Membranes were hybridized at 60 C for 16 h. cDNA- and cRNA-probed membranes were then washed in 2 × SSC/1% SDS for 10 min at room temperature at progressively higher wash stringencies to a maximum of 0.1 × SSC/0.1% SDS at 42–68 C for 30 min depending on the probe, followed by washes in 2 × SSC/0.1% SDS (10 min, room temperature) and 0.2 × SSC/0.1% SDS (20 min, 68 C) as previously described (33, 37). After phosphorimage analysis (Storm 850 Phosphor-Imager, Molecular Dynamics, Inc., Sunnyvale, CA), membranes were also subjected to autoradiography between two intensifying screens at −80 C for up to 10 days. The relative abundance of specific mRNA species in each tissue sample was quantified from either the phoshorimaged membrane or the autoradiograph using Phoretix Gel Analysis Software (NonLinear Dynamics, Newcastle upon Tyne, UK) within the linear range of the image or autoradiographic film (DuPont-Cronex). mRNA abundance was expressed as a fraction of the relative abundance of 18S ribosomal RNA (rRNA) to correct for variations in gel loading and efficiency of RNA transfer.

Probes

cDNAs encoding rat GR (1150-bp fragment in pBluescript, pGR14X) (32), 11βHSD1 (1265-bp fragment in pBluescript SK) (33), 11βHSD2 (1864-bp fragment in pCR3) (34), MR (513-bp fragment in pGEM4) (35), Na/K-ATPase α1 (332-bp fragment in Puc18) (36) and β1 (271-bp fragment in pIBI30) (37), and 18S ribosomal DNA (rDNA; 1070-bp fragment subcloned into pBluescript) (38) were donated by Drs Keith Yamamoto, Perrin White, Elise Gomez-Sanchez, Ronald Evans, Jerry Lingrel, and Ira Wool, respectively.

cDNA probes for 11βHSD1, 11βHSD2, Na/K-ATPase α1 and β1, and 18S were synthesized and radiolabeled with [^{32}P]deoxy-CTP (3000 Ci/mmol) by oligonucleotide random priming of the restriction endonuclease-excised cDNA fragment (10^9 cpm/μg DNA) using commercially available kits (Amersham Pharmacia Biotech) as previously described (29). An antisense GR cRNA probe was synthesized by T7 RNA polymerase-directed *in vitro* transcription from the 1150-bp cDNA fragment after linearization of the plasmid with *Xho*I as described previously (39). An antisense MR cRNA probe was similarly synthesized by SP6 RNA polymerase directed *in vitro* transcription from the 523-bp cDNA fragment after linearization of the plasmid with *Hin*dIII as previously described (39). The cRNA probes were radiolabeled by incorporation of [^{32}P]UTP (3000 Ci/mmol). The *in vitro* synthesis of cRNA probes employed transcription reagents and enzymes from Promega Corp. UK (Southampton, UK) and yielded probes comprising more than 90% full-length transcripts that were analyzed by electrophoresis on a denaturing 6% polyacrylamide/7 M urea gel as previously described (39).

SDS-PAGE and Western blot analysis

Stock protein samples were prepared for SDS-PAGE by the addition of an equal volume of protein sample buffer [62.5 mM Tris-HCl (pH 6.8), 4% SDS, 10% glycerol, 10% β-mercaptoethanol, and 0.2% bromophenol blue] and boiled for 10 min. For each tissue equal quantities of total protein (20–100 μg) were loaded and electrophoresed through an SDS-PAGE gel composed of a 4% stacking gel (pH 6.8) and 10% separating gel (pH 8.8). Separated proteins were transferred for 1 h at 0.8 mA/cm^2 onto nitrocellulose membrane (Amersham Pharmacia Biotech-Pharmacia) using a Trans-Blot semidry blotting apparatus (Bio-Rad Laboratories, Inc.). Membranes were blocked for nonspecific protein binding by incubation overnight at 4 C in Tris-buffered saline [TBS; 20 mM Tris-HCl (pH 7.4) and 200 mM NaCl] containing 5% nonfat milk. For detection of GR, membranes were incubated with polyclonal rabbit antimouse GR antisera (Autogen Bioclear, Santa Cruz Biotechnology, Inc., Salisbury, UK) diluted 1:200 with 1% milk/TBS, washed vigorously with TBS (three times, 20 min each time), and then incubated with a 1:2000 dilution of sheep antirabbit IgG horseradish peroxidase conjugate (Sigma-Aldrich Corp.). After additional washing with TBS (three times, 20 min each time), GR protein was visualized by autoradiography using the ECL-Plus chemiluminescent system (Amersham Pharmacia Biotech-Pharmacia). In all analyses a single species, approximately 97 kDa in size, was identified with negligible background signal. The relative abundance of GR protein expression in each tissue sample was quantified from the autoradiograph using Phoretix Gel Analysis Software (NonLinear Dynamics, Newcastle upon Tyne, UK) within the linear range of the autoradiographic film (DuPont-Cronex) and expressed as OD units in relation to those of a standard. In all protein analyses the standard refers to protein harvested from pooled tissue samples.

Statistics

All data were found to be normally distributed by Kolmogorov-Smirnov analysis and are presented as the mean ± SEM. Birth weight, systolic blood pressure, and tissue expression of GR, MR, 11βHSD1, 11βHSD2, and Na/K-ATPase α1 and β1 in each tissue were compared between the MLP and control groups by Student's *t* test, assuming unequal variance. One way ANOVAs were also employed to determine whether there was a significant effect of maternal undernutrition on offspring birth weight, blood pressure, and expression of these genes in a range of offspring tissues from before birth to well into adult life. Statistical analyses were performed using SPSS software (SPSS, Inc., Chicago, IL). $P < 0.05$ was considered significant. Analyses of imprecision for Northern and Western blot analyses revealed percent coefficient of variations within a gel of less than 10% and between gels of less than 14%.

Results

Effects of the MLP diet on offspring birth weight

In keeping with observations from previous studies (9, 40), maternal protein restriction throughout gestation from conception to term significantly reduced offspring birth weight (MLP, 5.41 ± 0.05 g; control, 5.86 ± 0.33 g; $P < 0.05$). These data represent measurements of 114 offspring from 12 litters born to MLP-fed dams and 139 offspring from 12 litters born to control-fed dams. The effect was equally evident in the male and female offspring, and there was no significant effect of the maternal diet on litter size, length of gestation, off-

spring sex ratio, or offspring viability and weight gain (data not shown). The effects of the MLP diet on maternal weight gain throughout pregnancy, fetal growth trajectory, and placental size at mid- to late gestation (day 14) and close to term (day 20) have been reported previously (9, 40)

Effects of the MLP diet on offspring systolic blood pressure

Offspring that had been exposed to maternal protein restriction *in utero* exhibited significantly higher systolic blood pressures that were 25–35 mm Hg above those in control offspring from when measurements commenced at 4 weeks of age (9). Thus, systolic blood pressure (mm Hg, mean ± SEM) for MLP *vs.* control offspring was 135 ± 5 *vs.* 111 ± 3 ($P = <0.001$) at 4 weeks, 140 ± 4 *vs.* 116 ± 3 ($P = <0.001$) at 8 weeks, and 142 ± 4 *vs.* 115 ± 5 ($P = <0.001$) at 12 weeks.

The MLP diet resulted in increased blood pressure in the offspring that persisted in both males (n = 96) and females (n = 96) throughout adult life. Thus, this pattern was similarly evident at other age points in older offspring up to 20 weeks of age, when measurements halted. This confirms previous observations of life-long programmed hypertension in this rat model (9). In further accordance with previous studies (9), comparative studies of offspring up to 20 weeks of age revealed no effect of gender (two-way ANOVA) on the blood pressure programming effects of the MLP diet. However, similar analyses for the effect of postnatal age revealed an age-related increase in the blood pressure increment between the two groups ($P < 0.05$), *i.e.* the difference between blood pressures in the MLP *vs.* control offspring increased with age ($P < 0.05$).

Effects of the MLP diet on the expression of 11βHSD isoforms in the placenta and fetal and neonatal offspring tissues

On day 20 of gestation (term = 22 days), levels of 11βHSD2 mRNA were markedly lower in the placentas from rats fed the MLP diet throughout pregnancy compared with those in the control-fed animals (Fig. 1, A and B). The inhibitory effect of the MLP diet on placental 11βHSD2 expression was also evident on day 14 of gestation, although the difference was slightly less marked (Fig. 1, A and B). There was no effect of the MLP diet on placental expression of 11βHSD1 on day 20, and levels were undetectable on day 14 (data not shown). Reduced levels of 11βHSD2, but unchanged levels of 11βHSD1, expression were also evident in late gestation (day 20) fetal tissues from MLP offspring, in which expression of both isoforms was detectable, *e.g.* in the lung (Fig. 2, A and B).

In both neonatal and adult offspring detectable levels of 11βHSD2 mRNA expression were confined to mineralocorticoid target tissues, such as the kidney (Figs. 3 and 6). In keeping with observations in the placenta, the MLP diet resulted in attenuated levels of 11βHSD2 expression in the offspring kidney from birth (Fig. 3). As described in detail below, this effect persisted through early juvenile life and into adulthood (Fig. 6). In contrast, levels of 11βHSD1 expression in neonatal offspring kidney were unaffected by the MLP diet (Fig. 3).

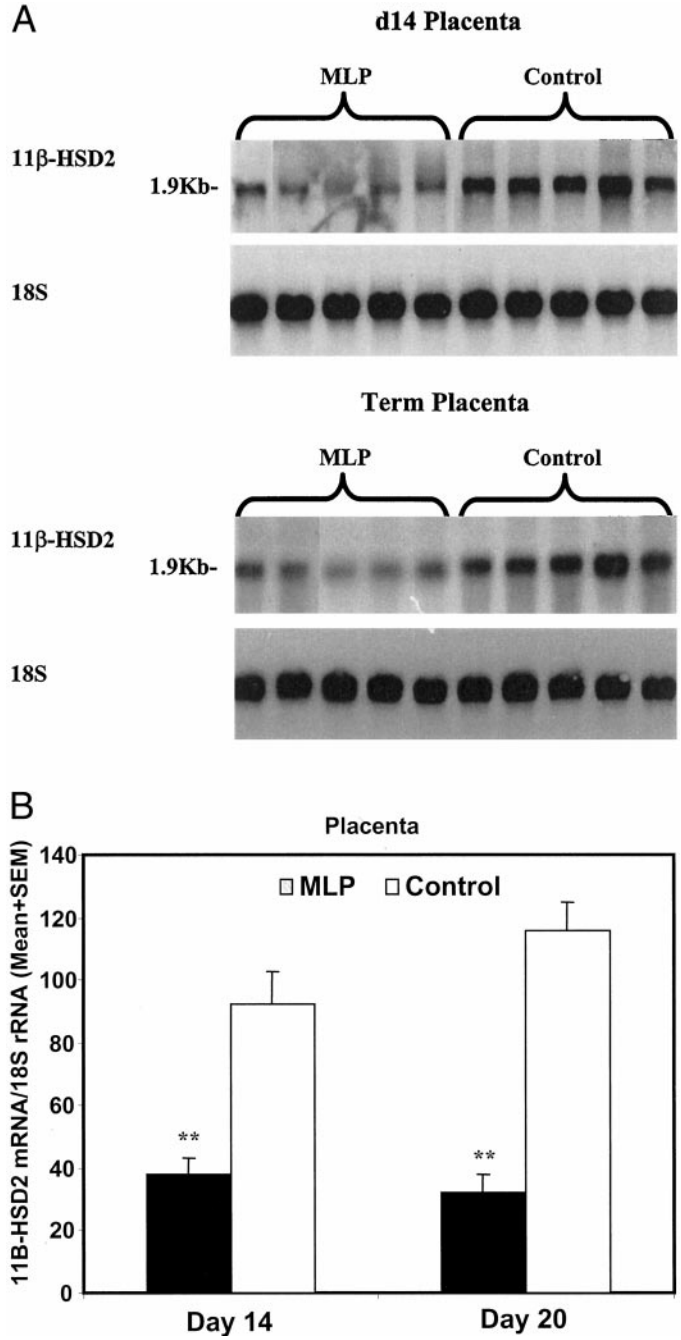

FIG. 1. A, Northern blot analysis of 11βHSD2 mRNA (1.9 kb) expression in total RNA isolated from representative samples of placenta on days 14 and 20 of gestation (term = 22 days) from MLP-fed rats (n = 8) *vs.* control-fed rats (n = 8). RNA in each lane has been pooled from more than three aliquots of tissue from each offspring liver. Even loading of the gel was confirmed by probing with a rDNA probe for 18S rRNA. B, Histogram depicting levels of 11βHSD2 mRNA in relation to those for 18S rRNA in day 14 and day 20 placental tissue from rats fed the MLP diet throughout pregnancy *vs.* control-fed rats (mean ± SEM). **, $P < 0.001$.

Effects of the MLP diet on the expression of GR and MR in fetal and neonatal offspring tissues

Levels of GR expression were significantly greater in peripheral tissues from both late gestation (day 20) fetal and

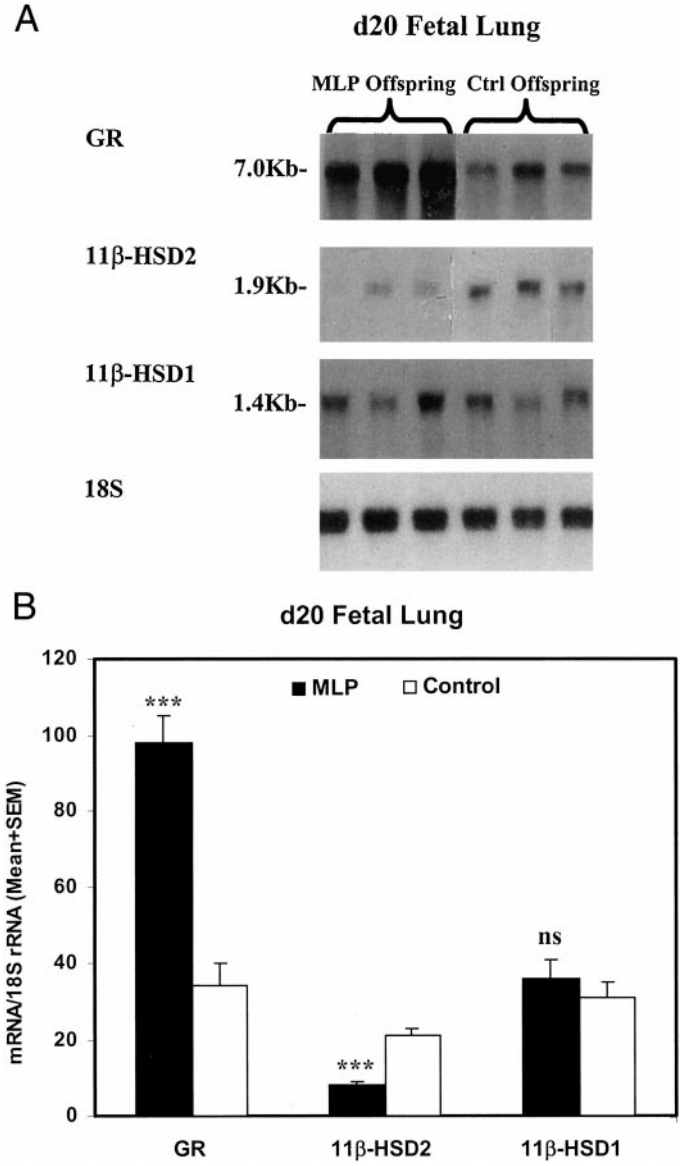

FIG. 2. A, Northern blot analysis of GR mRNA (7.0 kb), 11βHSD2 mRNA (1.9 kb), and 11βHSD1 mRNA (1.4 kb) expression in total RNA isolated from the lung of MLP *vs.* control fetal offspring on day 20 of gestation. RNA in each lane has been pooled from more than three aliquots of tissue from each offspring organ. The blot shows mRNA expression in the lung from representative day 20 fetal offspring; each from separate litters in the MLP group and the control group. Even loading of the gel was confirmed by probing with a rDNA probe for 18S rRNA. B, Histogram depicting levels of GR, 11βHSD2, and 11βHSD1 mRNA expression in relation to those of 18S rRNA in fetal offspring from rats fed the MLP diet throughout pregnancy (more than one offspring from each of a total of eight litters, *i.e.* a total of at least eight offspring generated from eight separate litters) *vs.* control fed rats (more than one offspring from each of a total of seven litters, *i.e.* a total of at least seven offspring generated from seven separate litters; mean ± SEM). **, $P < 0.001$.

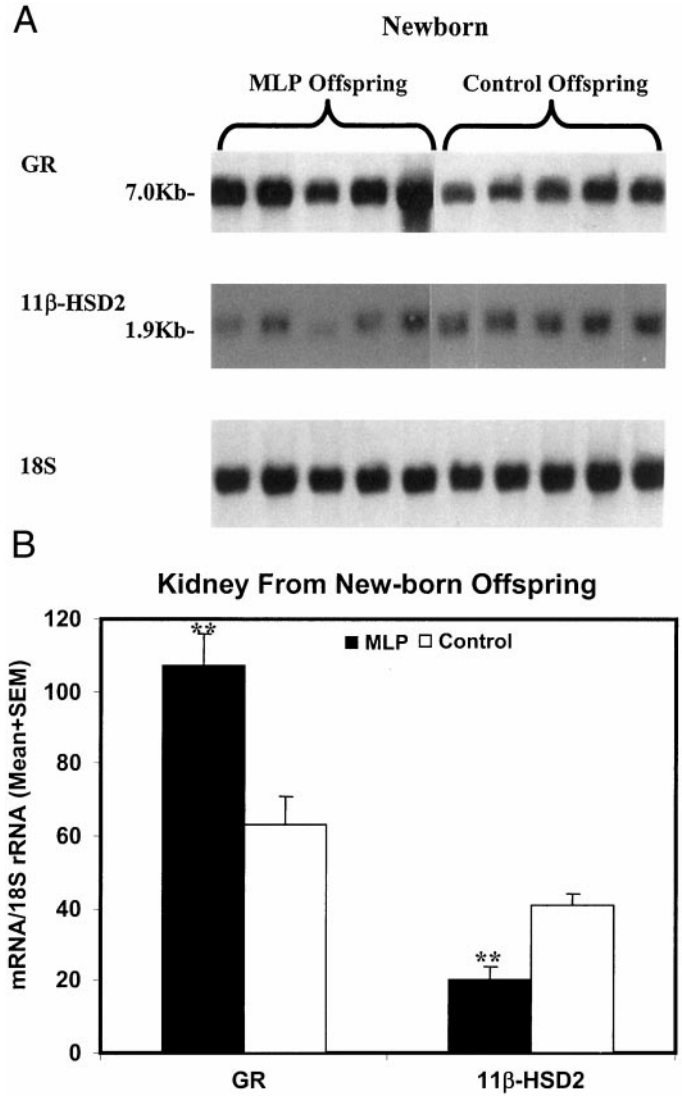

FIG. 3. A, Northern blot analysis of GR mRNA (7.0 kb) and 11βHSD2 mRNA (1.9 kb) expression in total RNA isolated from the kidney of MLP *vs.* control offspring at birth. RNA in each lane has been pooled from more than three aliquots of tissue from each offspring organ. The blot shows mRNA expression in the lung from representative newborn offspring, each from a separate litter in each diet group. Even loading of the gel was confirmed by probing with a rDNA probe for 18S rRNA. B, Histogram depicting levels of GR and 11βHSD2 mRNA expression in relation to those of 18S rRNA in the kidney from newborn offspring from rats fed the MLP diet throughout pregnancy (more than one offspring from each of a total of five litters, *i.e.* a total of at least five offspring generated from five separate litters) *vs.* control fed rats (more than one offspring from each of a total of five litters, *i.e.* a total of at least five offspring generated from five separate litters; mean ± SEM). **, $P < 0.001$.

neonatal offspring that had been exposed to maternal protein restriction compared with those in fetal and neonatal offspring from control diet-fed rats. For example, levels of GR mRNA expression were approximately 2-fold higher in the day 20 fetal lung and neonatal kidney from MLP *vs.* control offspring (Figs. 2 and 3). The stimulatory effects of maternal protein restriction during pregnancy on fetal and neonatal offspring tissue expression of GR were also evident at the protein level. Figure 4 shows a 2-fold greater abundance of GR protein expression in the kidney and lung (representative of classical glucocorticoid and mineralocorticoid target tissues) from MLP *vs.* control offspring at birth. Levels of MR expression were unaffected by the maternal diet in the kidney of MLP offspring and were undetectable in the lung.

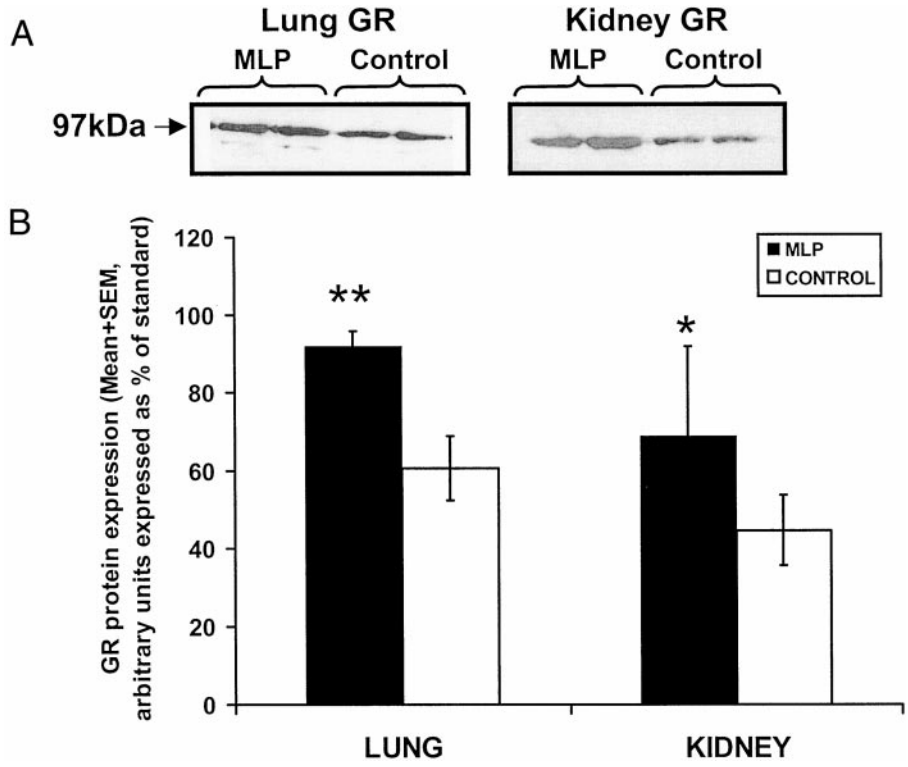

FIG. 4. A, Western blot analysis of GR protein (97 kDa) expression in total tissue protein isolated from the lung and kidney of fetal offspring on day 20 of gestation from rats fed the MLP diet throughout pregnancy *vs.* control fed rats. Protein in each lane has been pooled from more than three aliquots of tissue from each offspring organ. B, Histogram depicting levels of GR protein expression in lung and kidney from day 20 fetal offspring from rats fed the MLP diet (more than one offspring from each of a total of eight litters, *i.e.* a total of at least eight offspring generated from eight separate litters) throughout pregnancy *vs.* control-fed rats (more than one offspring from each of a total of eight litters, *i.e.* a total of at least eight offspring generated from eight separate litters). Levels of GR protein, measured as OD units, are expressed as a percentage of that in the standard (mean ± SEM). The standard refers to protein harvested from pooled tissue samples. *, $P < 0.05$.

Effects of the MLP diet on renal GR, MR, and 11βHSD isoforms and corticosteroid-responsive Na/K-ATPase gene expression in offspring during postnatal life

Levels of GR mRNA and GR protein expression were markedly elevated in the kidneys from juvenile and adult offspring that had been exposed to maternal protein restriction during fetal life compared with those in the kidneys from control offspring (Figs. 5 and 6). In keeping with the stimulatory effects of the MLP diet on renal GR expression shortly after birth (Figs. 3 and 4), the abundance of GR protein expression was also approximately 2-fold greater in MLP *vs.* control offspring from preweaning (*i.e.* 2 weeks of age) through juvenile life and into adulthood (Fig. 5). Levels of GR mRNA expression were also persistently 2-fold higher in the kidney from MLP *vs.* control offspring. Northern blot analyses of the effects of the MLP diet on renal GR expression in 12-week-old offspring, which are representative of similar patterns of expression in the other age groups, are shown in Fig. 6.

In contrast, although the MLP diet had no effect on renal MR expression in the offspring (Fig. 6), it resulted in a marked decline in 11βHSD2 expression similar to that observed in the kidney at birth and in other feto-placental tissues. The inhibitory effect of the MLP diet on renal 11βHSD2 expression was also evident in offspring at other ages (data not shown). These prenatally programmed increases in GR expression and decreases in 11βHSD2 expression in the kidney from MLP offspring were accompanied by a concomitant 2- to 3-fold increase in the expression of corticosteroid-responsive Na/K-ATPase α1- and β1-subunits (Fig. 6).

Effects of the MLP diet on the abundance of GR and 11βHSD isoforms and corticosteroid-responsive Na/K-ATPase gene expression in other offspring tissues during postnatal life

The effects of the MLP diet on levels of GR expression in the offspring were tissue specific. The abundance of GR expression was markedly greater in classical glucocorticoid target tissues (but not in the heart or brain) from MLP *vs.* control offspring throughout adult life. Levels of GR protein were approximately 2-fold greater in the liver and 3-fold greater in the lung (Fig. 7) from MLP offspring compared with controls at 2 weeks of age. The potent programming effect of the MLP diet on GR expression in these tissues persisted from early life into adulthood, as demonstrated by quantitatively similar elevated levels of GR protein expression in both liver and lung from MLP *vs.* control offspring at 12 weeks of age (Fig. 7).

The MLP diet-induced programming of elevated GR protein expression was underpinned by programming of similarly elevated levels of GR mRNA expression in these tissues. In the lung from 12-week-old offspring, levels of GR mRNA expression were increased by 2- to 3-fold in those offspring that had been exposed to protein restriction *in utero* (Fig. 8). This pattern of programmed gene expression was also evident in 4-, 8-, 16-, and 20-week-old offspring (data not shown).

In keeping with observations in kidney, this was associated with unchanged levels of 11βHSD1 expression, but markedly increased levels of corticosteroid-responsive Na/K-ATPase α1- and β1-subunit mRNA expression (Fig. 8). 11βHSD2 expression was undetectable in liver and lung.

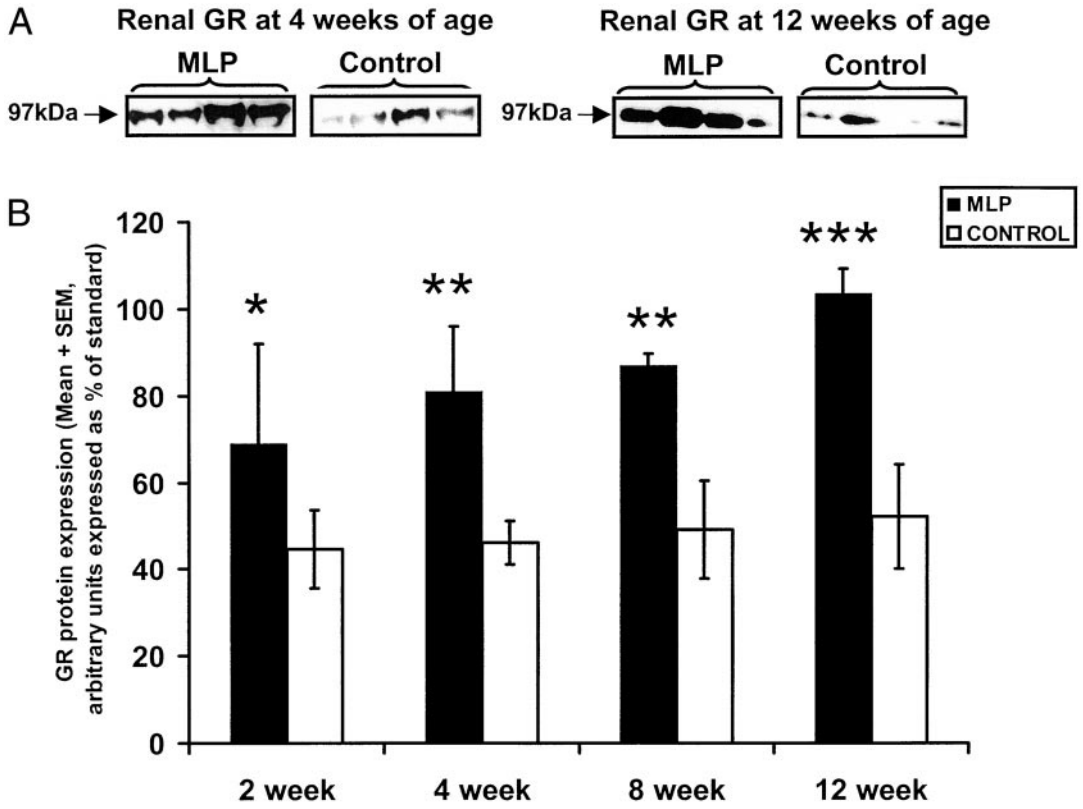

FIG. 5. A, Western blot analysis of GR protein (97 kDa) expression in total tissue protein isolated from representative samples of kidney from 4- and 12-week-old offspring born to rats fed the MLP diet throughout pregnancy *vs.* those born to control-fed rats. Protein in each lane has been pooled from more than three aliquots of tissue from each offspring organ. B, Histogram depicting levels of GR protein expression in the kidney from 2-, 4-, 8-, and 12-week-old offspring from rats fed the MLP diet throughout pregnancy *vs.* control-fed rats. Analyses were performed on more than 6 offspring from more than 3 separate litters (*i.e.* a total of at least 18 offspring) per diet group at each of the time points (*i.e.* 2, 4, 8, and 12 weeks postnatally). A similar pattern of expression is also evident in offspring aged 16–20 weeks (data not shown). Levels of GR protein, measured as OD units, are expressed as a percentage of that in a standard. (mean ± SEM). The standard refers to protein harvested from pooled tissue samples. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Levels of expression of the GR and corticosteroid-responsive Na/K-ATPase α1- and β1-subunits and phenylethanolamine-*N*-methyl transferase (metabolizes noradrenaline to adrenaline) were also persistently elevated in the adrenal gland from MLP offspring compared with controls (Copin, N., and C. B. Whorwood, unpublished observations).

In contrast with the MLP programmed up-regulation of GR expression in classical glucocorticoid target tissues and the absence of any effect in the heart, levels of GR mRNA and protein expression were markedly decreased in the hypothalamus from MLP *vs.* control offspring (Fig. 9). This was accompanied by a concomitant 50% decline in corticosteroid-responsive Na/K-ATPase α1-subunit mRNA expression (Fig. 9). As in other tissues, levels of 11βHSD1 expression in offspring hypothalami were unaffected by the MLP diet during pregnancy. Expression of 11βHSD2 and Na/K-ATPase β1-subunit was not detectable in this tissue.

Discussion

The present study demonstrates that mild restriction of maternal protein intake programs tissue-specific changes in the expression of genes that contribute to the regulation of blood pressure. Increased expression of the GR and corticosteroid-responsive Na/K-ATPase α1- and β1-subunits in key peripheral target tissues, such as the kidney, liver, and lung was observed. Furthermore, there were decreased levels of GR and Na/K-ATPase gene expression in the hypothalamus, a central tissue that mediates negative feedback regulation of hypothalamic-pituitary-adrenal axis activity. An additional finding from this study is that this relatively subtle isocaloric maternal dietary manipulation also programs markedly attenuated levels of 11βHSD2 expression in the kidney. Indeed, the prenatal programming effect of the MLP diet appears to be not only tissue specific, but also confined to the regulation of corticosteroid hormone action governed by the GR and 11βHSD2 rather than that dictated by the MR and 11βHSD1.

The MLP diet programming effects on GR, 11βHSD2, and Na/K-ATPase gene expression persist from before birth, through juvenile life, and into adulthood. As such, they parallel the effects of the MLP diet on birth weight and the subsequent manifestation of hypertension seen in the MLP offspring generated in this study and accord with earlier observations of the effects of maternal undernutrition on offspring birth weight and later hypertension described previously (8, 9). The significance of our data is that they reveal potential molecular mechanisms underlying the key role of glucocorticoid hormone action in the link between maternal

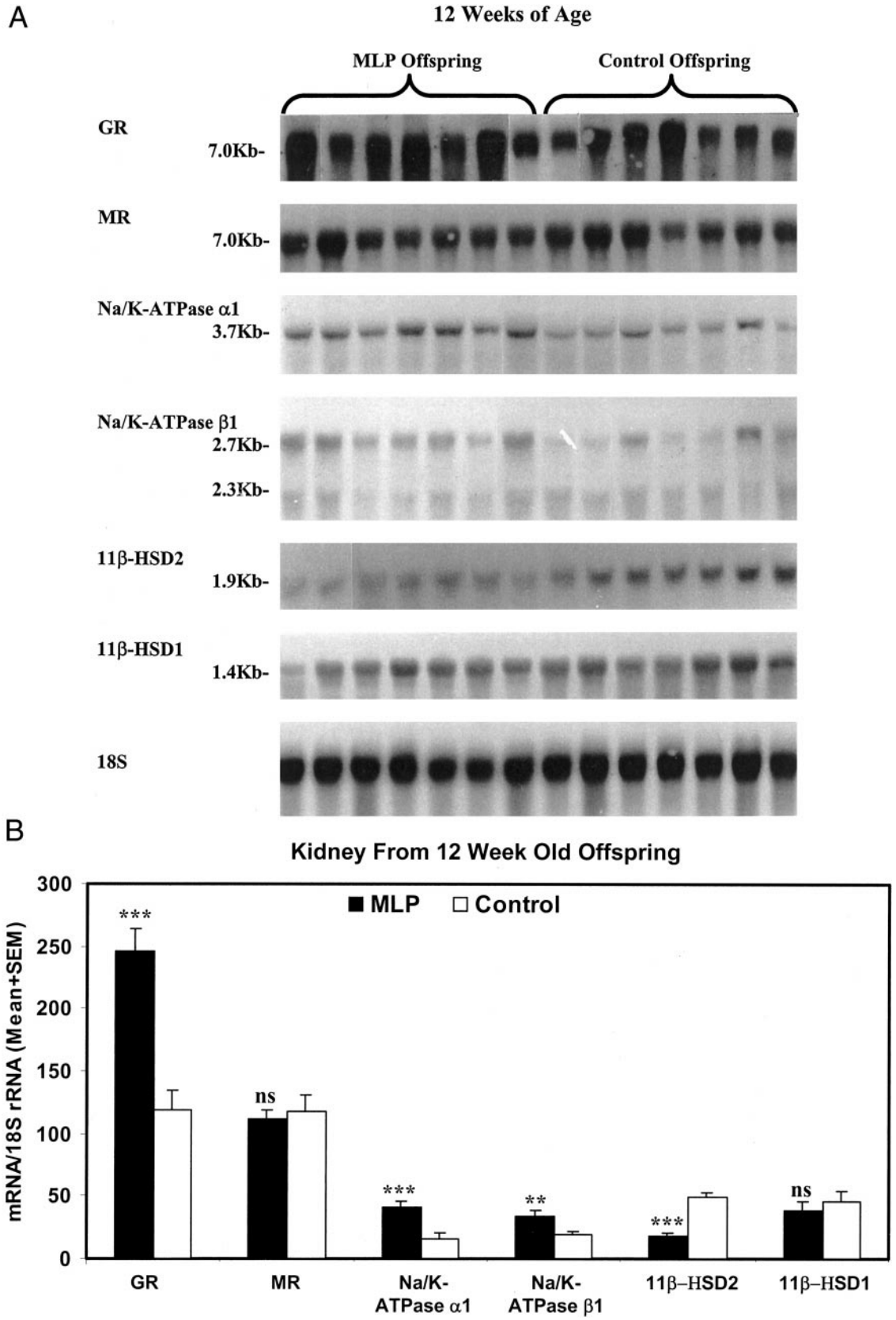

FIG. 6. A, Northern blot analysis of GR mRNA (7.0 kb), MR mRNA (7.0 kb), Na/K-ATPase α1 mRNA (3.7 kb), Na/K-ATPase β1 mRNA (2.7 and 2.3 kb), 11βHSD2 mRNA (1.9 kb), and 11βHSD1 mRNA (1.4 kb) expression in total RNA isolated from the kidney of MLP *vs.* control offspring at 12 weeks of age. RNA in each lane has been pooled from more than three aliquots of tissue from each offspring organ. The blot

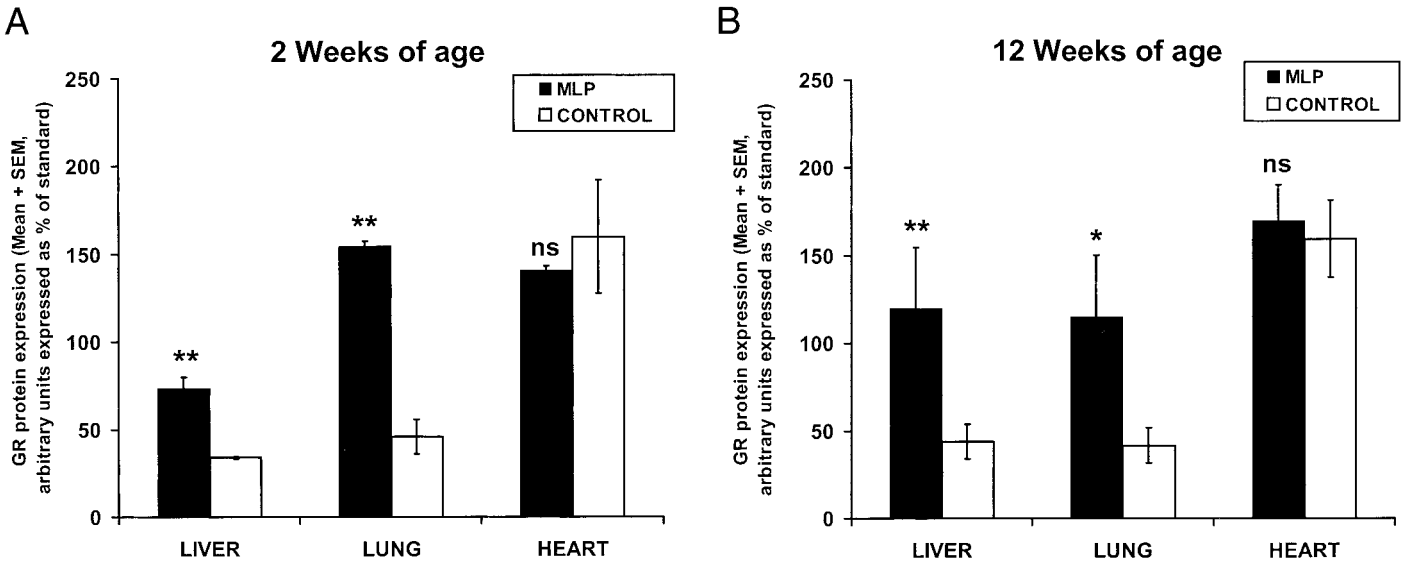

FIG. 7. Histograms showing data generated from representative Western blot analyses of GR protein (97 kDa) expression in the liver, lung, and heart from offspring of rats fed the MLP diet throughout pregnancy *vs.* control-fed rats at 2 weeks of age (A) and 12 weeks of age (B). Analyses were performed on more than 6 offspring from more than 3 separate litters (*i.e.* a total of at least 18 offspring) per diet group at both 2 and 12 weeks of age. A similar pattern of expression is also evident in offspring aged 16–20 weeks (data not shown). Levels of GR protein are expressed as a percentage of that in a standard (mean ± SEM). *, $P < 0.05$; **, $P < 0.01$.

nutrition, fetal growth retardation, and programming of hypertension that has recently been identified in animal models (10, 14, 40) and human populations (22).

We have previously shown that the programming effects of the MLP diet are dependent on maternal circulating glucocorticoids (14), which are likely to pass into the fetal circulation at an inappropriately high level as a result of the inhibitory effects of maternal protein restriction on placental 11βHSD2 activity (10). In normal physiology, this enzyme metabolizes active cortisol (or corticosterone in the rat) to inactive cortisone (11-dehydrocorticosterone in the rat) (24) and, as such, serves to protect the fetus from the deleterious effects of excess exposure to the higher levels of maternal glucocorticoid (13, 14, 26, 27). Indeed, two groups describing parallel increases in levels of 11βHSD2 mRNA expression and 11-dehydrogenase activity in fetal sheep kidneys throughout the latter half of gestation to term (31, 41) suggest that placental 11βHSD2 may also protect the renal MR from occupancy by the prepartum surge in fetal glucocorticoid levels that is necessary for parturition in this species. Data from the present study suggest that the inhibitory effect of the MLP diet on placental 11βHSD2 activity is mediated by attenuating the levels of 11βHSD2 gene transcription.

This nutrient/11βHSD2 gene interaction was evident not only in the placenta, but also in other fetal tissues in which this key enzyme is expressed. The previously described close correlation between levels of 11βHSD2 mRNA and activity (24, 31) suggests that the maternal diet effects on fetal tissue 11βHSD2 gene expression are likely to result in attenuated fetal tissue inactivation of glucocorticoid and hence greater levels of glucocorticoid hormone action in these tissues. As there is tissue-specific ontogeny of both GR and MR expression during late gestation in the rodent (42), a decline in feto-placental 11βHSD2 is likely to potentiate increased glucocorticoid binding to both receptors in those tissues in which they are coexpressed (*e.g.* kidney). Increased levels of corticosteroid-responsive Na/K-ATPase α1- and β1-subunit (29) expression in fetal and neonatal tissues from MLP offspring are consistent with this hypothesis.

The physiological importance of feto-placental 11βHSD2 expression, with respect to the role of glucocorticoid hormone action in mediating the programming effects of the MLP diet (10, 14), is highlighted by studies employing a pharmacological inhibitor of 11βHSD2, *i.e.* carbenoxolone (15). Maternal treatment with carbenoxolone results in increased fetal exposure to glucocorticoid-reduced fetal growth and programmed hypertension and insulin resistance in the offspring during later life (15). The adverse prenatal programming effects of maternal malnutrition during pregnancy on the blood pressure and insulin sensitivity of the offspring (8–10) can be mimicked in the rat and the sheep by maternal treatment with a synthetic glucocorticoid (*i.e.* dexamethasone) that is poorly metabolized by 11βHSD2 and, as such, is able to bypass the placental barrier (16, 28, 43).

In addition to the inhibitory effects of the MLP diet on 11βHSD2 gene expression, the present study suggests that

shows mRNA expression in the kidney from representative 12-week-old offspring, each from a separate litter in each diet group. Even loading of the gel was confirmed by probing with an rDNA probe for 18S rRNA. B, Histogram depicting levels of GR, MR, Na/K-ATPase α1, Na/K-ATPase β1, 11βHSD2, and 11βHSD1 mRNA expression in relation to those for 18S rRNA in kidney from 12-week-old offspring of rats fed the MLP diet throughout pregnancy (>1 offspring from each of a total of 15 litters, *i.e.* a total of at least 15 offspring generated from 15 separate litters) *vs.* control fed rats (>1 offspring from each of a total of 14 litters, *i.e.* a total of at least 14 offspring generated from 14 separate litters; mean ± SEM). **, $P < 0.01$; ***, $P < 0.001$.

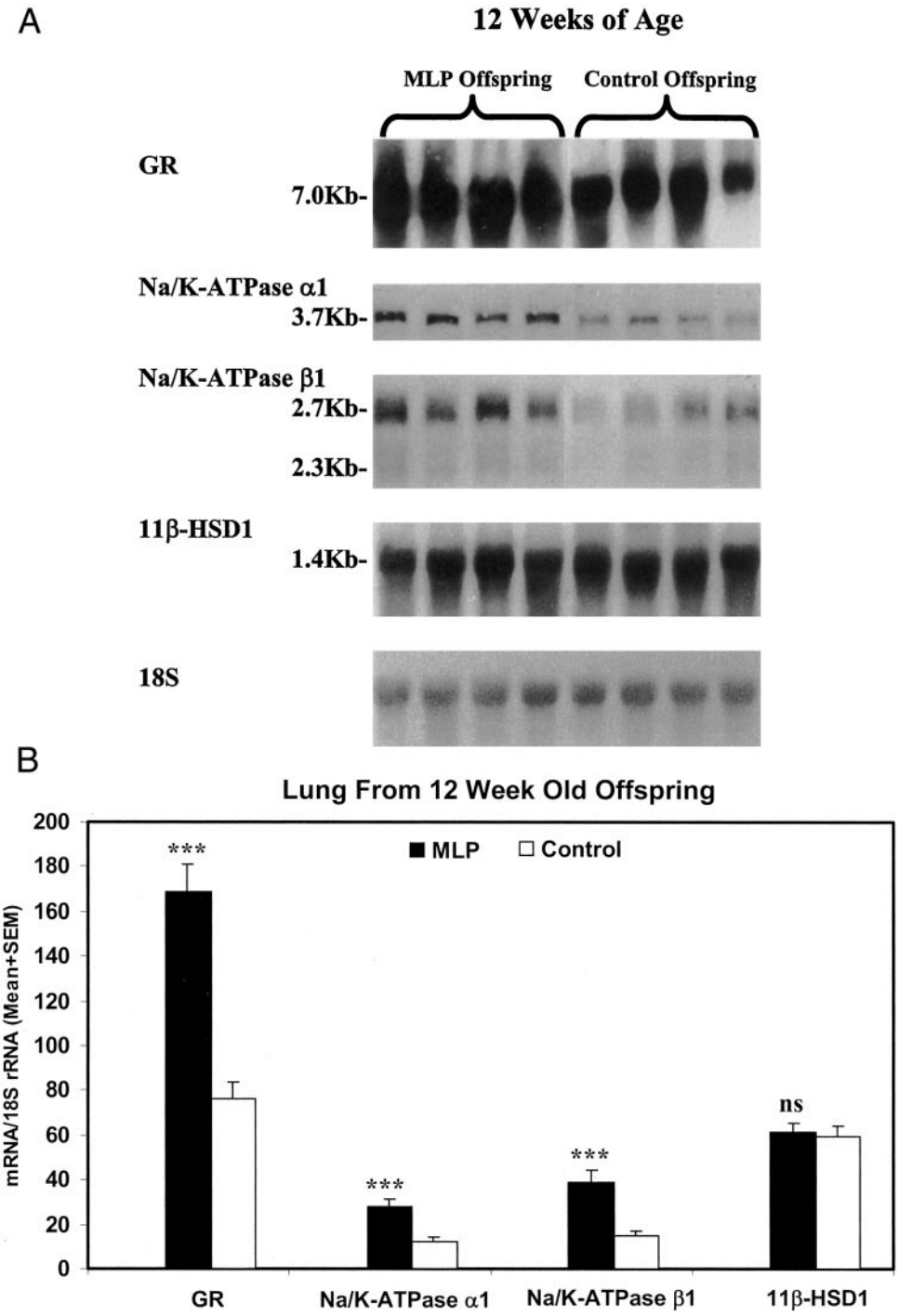

FIG. 8. A, Northern blot analysis of GR mRNA (7.0 kb), Na/K-ATPase α1 mRNA (3.7 kb), Na/K- ATPase β1 mRNA (2.7 and 2.3 kb), and 11βHSD1 mRNA (1.4 kb) expression in total RNA isolated from the lung of MLP *vs.* control offspring at 12 weeks of age. RNA in each lane has been pooled from more than 3 aliquots of tissue from each offspring organ. Blot shows mRNA expression in the lung from representative 12-week-old offspring; each from a separate litter in each diet group. Even loading of the gel was confirmed by probing with an rDNA probe for 18S rRNA. B, Histogram depicting levels of GR, Na/K-ATPase α1, Na/K-ATPase β1, and 11βHSD1 mRNA expression in relation to those for 18S rRNA in the lung from 12-week-old offspring from rats fed the MLP diet throughout pregnancy (>1 offspring from each of a total of 15 litters, *i.e.* a total of at least 15 offspring generated from 15 separate litters) *vs.* control-fed rats (>1 offspring from each of a total of 14 litters, *i.e.* a total of at least 14 offspring generated from 14 separate litters; mean ± SEM). ***, $P < 0.001$.

maternal dietary composition during pregnancy may also regulate fetal and neonatal tissue expression of the GR. Indeed, the stimulatory effects of the MLP diet on GR expression in classical glucocorticoid and mineralocorticoid target tissues (*i.e.* kidney and lung) are likely to have further enhanced the levels of glucocorticoid hormone action in these tissues beyond those that would be predicted to have resulted solely from the attenuated levels of 11βHSD2 expression. We report a clear interaction between maternal nutrient availability to the fetus and programmed expression of genes encoding key determinants of glucocorticoid hormone action in feto-placental tissue. The mechanisms underlying this interaction and the permanent establishment of these responses in the kidney and other tissues throughout adult life require further investigation. However, similar findings in an ovine model of maternal undernutrition programming of hypertension (40) suggest that this represents an important fundamental mechanism by which increased levels of glucocorticoid hormone action trigger programming events *in utero* that lead to disease in later life.

We have shown that the maternal dietary effects on the regulation of GR and 11βHSD2 gene expression during fetal

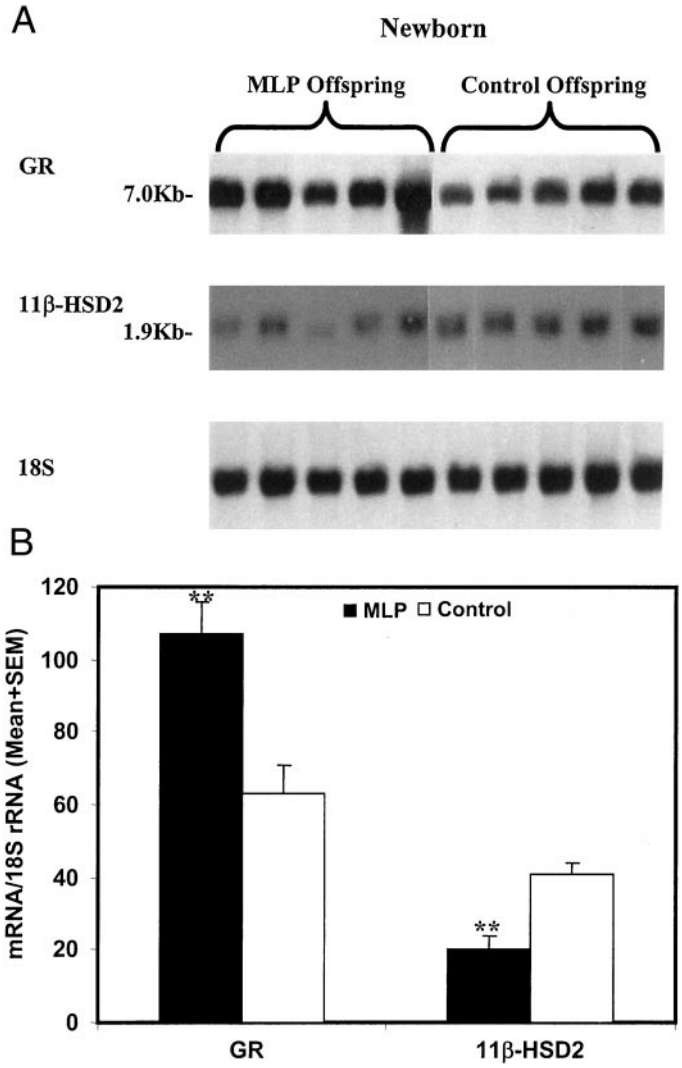

FIG. 9. A, Northern blot analysis of GR mRNA (7.0 kb), Na/K-ATPase α1 mRNA (3.7 kb), and 11βHSD1 mRNA (1.4 kb) expression in total RNA isolated from the hypothalamus of MLP *vs.* control offspring at 12 weeks of age. RNA in each lane has been pooled from more than 3 aliquots of tissue from each offspring organ. Blot shows mRNA expression in the hypothalamus from representative 12-week-old offspring; each from a separate litter in each diet group. Even loading of the gel was confirmed by probing with an rDNA probe for 18S rRNA. B, Histogram depicting levels of GR, Na/K-ATPase α1, and 11βHSD1 mRNA expression in relation to those of 18S rRNA in lung from 12-week-old offspring from rats fed the MLP diet throughout pregnancy (>1 offspring from each of a total of 15 litters, *i.e.* a total of at least 15 offspring generated from 15 separate litters) *vs.* control fed rats (>1 offspring from each of a total of 14 litters, *i.e.* a total of at least 14 offspring generated from 14 separate litters; mean ± SEM). ***, $P < 0.001$.

and neonatal life persist throughout adult life, *i.e.* levels of expression of these genes can be programmed in a tissue-specific manner by the nature of nutrient availability to the offspring before birth. Similarly, the permanent alterations in Na/K-ATPase subunit gene expression, which parallel those for the GR, strongly suggest that programmed increases in renal, hepatic, and pulmonary GR expression, as reported in this study, underpin concomitant increases in levels of glucocorticoid hormone action in these tissues. Conversely, programmed decreases in hypothalamic GR expression are likely to bring about persistently decreased sensitivity of this tissue to glucocorticoid regulation of trophic hormone release.

In support of our findings in the MLP offspring, treatment of pregnant rats with dexamethasone programs increased expression of GR and glucocorticoid-responsive phosphoenolpyruvate carboxykinase (the rate-limiting step in gluconeogenesis) in the liver (16) and also decreased expression of the GR in the hippocampal nuclei that mediate the central control of hypothalamic-pituitary-adrenal axis activity (28). The present study extends observations in this pharmacological model of programming to reveal that altered levels of GR expression and glucocorticoid hormone action are prenatally programmed by maternal nutritional status during pregnancy. This effect is evident in these and other important glucocorticoid target tissues in both the torso and central nervous system, including lung, adrenal medulla, and hypothalamus. It is unclear why altered levels of GR expression in the heart from these offspring were not similarly programmed by maternal protein restriction during pregnancy. Nevertheless, programmed increases in levels of GR expression in key peripheral glucocorticoid target tissues, in concert with diminished GR expression in central tissues are consistent with circulating ACTH and glucocorticoid hormone data, which suggest that the MLP diet programs hyperactivity of the hypothalamic-pituitary-adrenal axis.

The absence of a programming effect of the MLP diet on 11βHSD1 gene expression in either central or peripheral tissues from the offspring is in keeping with similar findings in both pharmacological rat models of programmed hypertension (15, 16) and also our recent observations in an ovine maternal undernutrition model of programmed hypertension (44). Given the previously established close correlation between levels of 11βHSD1 mRNA and 11-oxoreductase activity (24, 41, 45), this suggests that 11βHSD1 does not contribute to programming of altered levels of glucocorticoid hormone action in these tissues. The lack of a programming effect of undernutrition or synthetic glucocorticoids on the expression of 11βHSD1 in tissues with programmed elevation of GR expression is surprising given that 11βHSD1 expression is up-regulated by glucocorticoid (24, 45, 46) and expression of other glucocorticoid target genes is increased (16, 44). However, as regulation of 11βHSD1 by glucocorticoids occurs in a complex tissue- and temporal-specific manner (46) and is also initiated by other factors (24), these variables may contribute to maintaining unchanged levels of 11βHSD1 expression despite the programming of increased levels of GR-mediated glucocorticoid hormone action in these tissues.

In contrast with the widespread tissue distribution of 11βHSD1, abundant levels of 11βHSD2 expression are confined to mineralocorticoid target tissues such as the kidney (24). This is in keeping with the well established role of 11βHSD2 as a key protector of the renal MR from access by glucocorticoid, such that its specificity for aldosterone is maintained (24). The programmed decline in renal 11βHSD2 expression in the MLP offspring from before birth and into adulthood is in keeping with observations in sheep, in which

fetal exposure to nutrient restriction or chronic intermittent hypoxemia down-regulates renal 11βHSD2 expression in the fetal lamb (44, 47), an effect that has been shown to persist during later life despite restoration of nutrient availability (44). A prenatally programmed decline in renal 11βHSD2 expression may potentially enhance glucocorticoid availability to both the MR and the GR. Not only will this result in increased levels of glucocorticoid hormone action, but it is also likely to induce a state of apparent mineralocorticoid excess similar to that in patients with congenital or acquired deficiency of 11βHSD2 activity (24).

The well documented hypertensive effects of congenital or acquired 11βHSD2 deficiency in humans (24) suggest that reduced levels of 11βHSD2 expression in MLP offspring and those described in the ovine maternal undernutrition model (44) may contribute to the manifestation of hypertension seen in the offspring from both species (9, 12). In support of this, it has been shown that in the kidney and adrenal gland that pharmacologically induced and nutritionally programmed decreases in 11βHSD2 expression promote increased expression of glucocorticoid-responsive genes in the adrenal and both glucocorticoid- and mineralocorticoid-responsive genes in the kidney. These are known to have potent effects on sodium uptake, fluid-electrolyte homeostasis, and vascular tone (29, 44, 48). Programming of attenuated 11βHSD2 expression may, therefore, represent a fundamental mechanism linking intrauterine life with the manifestation of hypertension in later life.

In addition to being a measure of the levels of corticosteroid hormone action in the kidney and other tissues, Na/K-ATPase α1- and β1-subunit mRNA expression encodes an important transmembrane sodium pump, which is the principal mechanism for ATP-dependent maintenance of sodium and potassium electrochemical gradients across the cell membrane (49). Na/K-ATPase is the main driving force for net sodium reabsorption across renal tubular and distal colonic epithelia (49), and in other tissues, such as skeletal and cardiac muscle, it provides the electrolyte gradient required for mechanical function (49). Corticosteroid-induced up-regulation of Na/K-ATPase represents an important mechanism by which increased levels of glucocorticoid hormone action promote sodium retention. This, in turn, promotes fluid retention, resulting in raised blood pressure as a consequence of maintaining fluid-electrolyte homeostasis (49). The prenatal programming of increased levels of Na/K-ATPase gene expression in the kidney from MLP offspring may, therefore, contribute significantly to mechanisms mediating the glucocorticoid-dependent programming of hypertension that has been described in these animals (14).

The potent effects of glucocorticoids with respect not only to fetal growth and development (17), but also to induction of hypertension, insulin resistance, and glucose intolerance are well documented (18–20). Our findings, therefore, indicate a plausible molecular basis for the programming and manifestation of hypertension in the MLP rat model in which altered levels of glucocorticoid hormone action in the kidney appear to play a key role. These data also suggest molecular mechanisms by which the MLP diet programs the activity of the hypothalamic-pituitary-adrenal axis, impaired glucose tolerance, and insulin resistance in the offspring during adult life (11). This would occur by potentiation of both increased levels of glucocorticoid hormone action in peripheral tissues and decreased glucocorticoid hormone action in central tissues regulating negative feedback control of the adrenal.

In the rat MLP model and the ovine maternal undernutrition model of programmed hypertension (9, 12), the mild manipulations of dietary composition and calorie/protein restriction and of timing and duration through pregnancy (9, 12, 40, 44) represent close approximations to the suboptimal levels of nutrition during human pregnancy that similarly alter patterns of fetal growth and program raised blood pressure and increased CVD risk in populations worldwide (1–3, 50). Furthermore, these analyses were performed at postnatal ages similar to those employed in other animal model investigations (8–17) and are broadly equivalent to those reported in more recent human epidemiological studies (1, 3). It remains to be seen whether the programming mechanisms identified in these animal models also contribute to the mechanisms linking events during intrauterine life and the programming of hypertension, insulin resistance, and other adverse risk factors for CVD in human populations, in which there is now compelling evidence that alterations of glucocorticoid hormone action play an important role.

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