0013-7227/01/$03.00/0
Endocrinology

Vol. 142, No. 7
Printed in U.S.A.

Maternal Undernutrition during Early to Midgestation Programs Tissue-Specific Alterations in the Expression of the Glucocorticoid Receptor, 11β-Hydroxysteroid Dehydrogenase Isoforms, and Type 1 Angiotensin II Receptor in Neonatal Sheep*

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ABSTRACT

We have investigated the effects of maternal nutrient restriction in the sheep during the period of rapid placental growth (*i.e.* 28–77 days gestation; term = 147 days) on feto-placental growth and expression of the glucocorticoid receptor (GR), types 1 and 2 11β-hydroxysteroid dehydrogenase (11βHSD1, 11βHSD2), and types 1 and 2 angiotensin II receptor (AT1, AT2) in fetal and neonatal offspring. Ewes (n = 63) of similar age, body weight, and body composition were randomly allocated to a nutrient-restricted (NR) group in which they consumed 3.2 MJ/day metabolizable energy (ME; equivalent to 50% of predicted requirements) or to a control group in which they consumed 6.7 MJ/day ME (equivalent to 110% of predicted requirements). After 77 days gestation, ewes from both dietary groups consumed close to 100% of ME requirements up to term. Newborn offspring of NR ewes were of similar body weight, but had increased crown-rump length, greater placental weight, and increased placental/body weight ratio ($P < 0.01$) compared with controls. Their kidneys were heavier ($P < 0.05$), but shorter in length, with increased ratios of transverse width to length ($P < 0.001$).

GR messenger RNA (mRNA) expression in neonatal offspring from NR ewes was increased in adrenal, kidney, liver, lung, and perirenal adipose tissue ($P < 0.01$). Conversely, 11βHSD1 mRNA expression was unaffected, except in perirenal adipose tissue, where it was higher in lambs born to NR ewes ($P < 0.01$). 11βHSD2 mRNA expression was decreased in adrenals and kidney ($P < 0.001$). Maternal NR also resulted in significantly increased AT1 expression in those tissues in which expression of GR was increased and/or 11βHSD2 was decreased, *i.e.* adrenals, kidney, liver, and lung. AT2 expression was unaffected by maternal NR. Although 11βHSD2 mRNA was undetectable in term placenta, it was abundant in midgestation placenta and was lower after maternal NR ($P < 0.001$). There was close agreement between levels of 11βHSD enzyme (*i.e.* 11β-dehydrogenase and 11-oxoreductase) activities and abundance of 11βHSD1 mRNA and 11βHSD2 mRNA expression.

The persistence of tissue-specific increases in the expression of GR, 11βHSD1 and AT1 and decreases in the expression of 11βHSD2 in adrenals and kidney in newborn offspring in response to a defined period of maternal nutrient restriction during early to midgestation suggests that gene expression has been programmed by nutrient availability to the fetus before birth. These data suggest key potential mechanisms by which maternal nutrition prenatally programs physiological pathways, such as the renin-angiotensin system, in the offspring that may lead to raised blood pressure and other cardiovascular disease risk factors in later life. (*Endocrinology* **142:** 2854–2864, 2001)

ROBUST EPIDEMIOLOGICAL evidence from populations worldwide indicates that the nutritional and hormonal environment encountered by the fetus is a strong determinant not only of fetal growth but also of cardiovascular disease (CVD) risk in later life. These associations between reduced birth weight and increased placental to birth weight ratio with the metabolic syndrome, type 2 diabetes, and subsequent premature mortality from heart disease are independent of adult life style risk factors such as obesity (1, 2).

The strongest associations between intrauterine life and increased prevalence of CVD risk factors relate to the effects of transient periods of maternal undernutrition during pregnancy on patterns of fetal growth and placental size rather than to low birth weight *per se* (3). This has led to the hypothesis that suboptimal nutrition during discrete periods of pregnancy permanently modifies or programs (*i.e.* results in persistent changes beyond the immediate period of nutrient restriction) fetal morphology, metabolism, and blood pressure regulation pathways. As a consequence and in association with maladaption to the postnatal environment, they confer greater risk of metabolic and cardiovascular disease in adult life (4). These epidemiological data are strongly supported by experimental animal studies. In rodents, both severe nutrient restriction to the fetus, through either uterine artery ligation (5) or major calorific restriction (6), and mild undernutrition arising from a maternal low protein diet during part or all of gestation result in offspring with low body weight and/or disproportionate body size at birth, which have elevated blood pressure (6, 7) and dysregulation of glucose metabolism in later life (8).

Received November 1, 2000.
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* This work was supported by the National Kidney Research Fund and the Research Strategy Committee of the University of Southampton.

The precise molecular mechanisms underlying the programming of adult disease by maternal undernutrition are unknown. However, recent experimental studies strongly suggest that fetal overexposure to maternal glucocorticoids (9–12) triggers programming events *in utero* that establish persistent increases in glucocorticoid action throughout life and hypertension (11–13). Glucocorticoids are potent regulators of fetal growth and development (14) and increase blood pressure by potentiating tissue sensitivity to vasoactive hormones (15, 16). They also regulate components of the renin-angiotensin system (RAS), including the type 1 angiotensin II receptor (AT1) (17), which are expressed at high levels during fetal and neonatal life (18). Glucocorticoids also promote gluconeogenesis and antagonize the actions of insulin (19).

Tissue sensitivity to glucocorticoid is regulated predominantly by intracellular expression of the glucocorticoid receptor (GR) and isoforms of 11β-hydroxysteroid dehydrogenase (11βHSD1, 11βHSD2) at the level of gene transcription. 11βHSD1 acts predominantly as an 11-oxoreductase, catalyzing the conversion of cortisone to bioactive cortisol and as an intracellular amplifier of glucocorticoid access to the GR (21, 22). Conversely, 11βHSD2 behaves as an 11β-dehydrogenase, catalyzing the inactivation of cortisol to cortisone, and thereby maintains the specificity of the mineralocorticoid receptor for aldosterone (22). In addition, 11βHSD2 expression in feto-placental tissues is thought to protect the fetus from the deleterious effects of excess glucocorticoid (9, 15, 22). Recent studies in the rat suggest that maternal undernutrition may increase fetal exposure to glucocorticoids, thereby impairing fetal growth and programming hypertension and glucose intolerance (10, 11) through inhibition of placental 11βHSD2 gene expression (9).

The close similarities between sheep and humans with respect to fetal growth and development, physiology, and ontogeny of endocrine pathways has prompted investigations into the effects of maternal nutrition on the resulting offspring in this species (23–25). The long gestation period in the sheep has also enabled more precise exploration of the existence of discrete windows of sensitivity to the programming effects of maternal undernutrition and maternal treatment with glucocorticoids on offspring blood pressure and hypothalamic-pituitary-adrenal axis activity (12, 23). Recent studies suggest that the cardiovascular sequelae of brief maternal nutrient restriction on placental size and birth dimensions, but not birth weight, observed in human populations (1, 4, 26) can be closely modeled in sheep (23–25). In the present study we describe for the first time the effects of nutrient restriction between early to midgestation in singleton-bearing pregnant ewes on the expression of GR, 11βHSD1, 11βHSD2, and glucocorticoid-responsive angiotensin receptors (18, 19) in fetal and neonatal tissues.

Materials and Methods

Animals and maternal dietary manipulation

This investigation comprised two separate studies with a total of 63 Welsh Mountain ewes of similar age (median, 3 yr), body weight (mean ± SEM, 36.14 + 0.85 kg), and body composition (2.7 ± 0.2 arbitrary units) within a scale of 0–5 (with 0 being very thin and 5 being grossly fat) (24, 25). This breed was chosen because it is able to withstand being fed a diet below energy requirements without loss of pregnancy and because it normally produces singleton fetuses. Indeed, only two pregnancies in this study resulted in twin lambs, which, as a result of interfetus competition for nutrients that was not present in singleton pregnancies, were excluded from analyses. Estrous activity was determined using a vasectomized ram that marked ewes in estrous with a colored dye on the rump. Timed matings were then established from the last date of observed estrus using one of two Texel rams.

Dietary manipulation commenced in the pregnant ewes at 28 days gestation. At this time, the ewes were individually housed to enable precise monitoring of food intake. The metabolizable energy (ME) requirement for each animal was calculated according to its body weight, taking into account requirements for both ewe maintenance and growth of the conceptus on the basis of producing a 4.5-kg lamb at term (27). Full dietary details have been previously published (25). Briefly, ewes were randomly allocated into either the nutrient-restricted (NR) group, which consumed 3.2 MJ/day ME (equivalent to 50% of ME requirements), or the control group, which consumed 6.7 MJ/day ME (equivalent to 110% of ME requirements) between 28 and 77 days gestation. As ME requirements increase during gestation, associated with growth of the conceptus, feed was adjusted fortnightly throughout the experimental period until day 77 of gestation. The diet comprised chopped hay that had an estimated ME content of 7.91 MJ/kg dry matter and a crude protein content of 69 g/kg dry matter and a barley-based concentrate that had an estimated ME content of 11.6 MJ/kg dry matter and a crude protein content of 162 g/kg dry matter. The proportion of hay to concentrate feed was approximately 3:1. All diets contained adequate minerals and vitamins (24). At 42 days gestation, ewes were confirmed as being pregnant with a single fetus using ultrasound scanning.

In study A, five ewes from each diet group were killed at 77 days gestation by lethal iv administration of barbiturate (100 mg/kg pentobarbital-sodium; *i.e.* Euthatal, PMB Animal Health, UK). Fetuses were delivered by cesarean section (24), and the lambs were immediately killed by iv barbiturate (as above). In a separate study (study B), nutrition was restored to the remaining 51 ewes from both nutrition groups on day 77 of gestation such that they consumed approximately 7.2 MJ/day ME (close to 100% of ME requirements) up to term (147 days). This was achieved by additional increases in the amount of feed provided at 100 days (*i.e.* an extra 14 g concentrate and 60 g hay) and 120 days (*i.e.* an extra 24 g concentrate and 100 g hay) gestation in accordance with increasing ME requirements (27). There was no difference in food intake or body weight between groups over this period (data not shown). Two ewes that had been NR aborted at approximately 120 days gestation and therefore were excluded from the study. At 144–146 days gestation all lambs were delivered by cesarean section (24) to remove the potentially confounding variable of stress and its endocrine sequelae that arise from natural parturition. Neonatal lambs were killed by iv barbiturate within 6 h of postnatal life. This generated 29 neonatal offspring from NR ewes and 20 from control ewes.

Tissue collection

In both studies fetal (day 77) and neonatal (days 144–146) lambs were weighed, and crown-rump length was measured before excision and weighing of adrenal glands, heart ventricles, kidney, liver, lung, and perirenal adipose tissue as well as hypothalamus and pituitary. In neonatal lambs the dimensions of both left and right kidneys were measured across transverse and longitudinal planes (28). Each ewe was then killed by iv barbiturate. After removal of the entire uterus, each placentome was excised, weighed (to determine total placental weight), and separated into fetal and maternal components by gently pulling apart the two tissues (25). Organs were excised from each lamb, weighed, snap-frozen in liquid nitrogen, and stored at −80 C for molecular analyses. All operating procedures were performed under Home Office approval as designated by the Animals (Scientific Procedures) Act (1986).

Northern blot analysis

Total RNA was extracted from fetal and neonatal sheep tissues using a modification of the single step acidified phenol-chloroform extraction method involving the use of Tri-Reagent (Sigma-Aldrich Corp., Poole,

UK) or RNAzol B (Biogenesis, UK) as previously described (29, 30). Total RNA was extracted, and integrity and quantification were assessed by comparison with RNA mol wt markers (Amersham Pharmacia Biotech, Little Chalfont, UK) coelectrophoresed in an ethidium bromide/agarose gel and by UV spectrophotometric absorbance at 260 nm.

Northern blot analyses were performed as previously described (29, 30). Briefly, total RNA from each tissue was electrophoresed through an agarose (1.5%)/formaldehyde (15%)/3-[*N*-morpholino]propanesulfonic acid (MOPS) gel and transferred in 20 × SSC (1 × SSC = 150 mm sodium chloride and 15 mm trisodium citrate) onto Hybond N^+ nylon membranes (Amersham Pharmacia Biotech) by capillary action. After UV cross-linking, membranes were prehybridized in hybridization buffer comprised of 50% deionized formamide, 4 × SSPE (20 × SSPE = 3.6 m sodium chloride, 0.2 m sodium dihydrogen phosphate, and 0.02 m EDTA, pH 8.0), 5 × Denhardt's solution (0.02% Ficoll, 0.02% polyvinylpyrrolidone, and 0.1% BSA), 10% dextran sulfate, 1% SDS, 0.5% Blotto (10% nonfat milk and 0.02% sodium azide), and 100 μg/ml denatured salmon sperm DNA and subsequently hybridized at 42 C for complementary DNA (cDNA) probes or 60 C for complementary RNA (cRNA) probes for 16 h. Membranes were then washed in 2 × SSC/1% SDS for 10 min at room temperature and subsequently at progressively higher wash stringencies to a maximum of 0.1 × SSC/0.1% SDS between 42–68 C for 30 min depending on the probe.

After PhosphorImager analysis (Storm 850 Phosphor-Imager, Molecular Dynamics, Inc., Palo Alto, CA) membranes were subjected to autoradiography at −80 C for up to 10 days. The relative abundance of specific messenger RNA (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) and expressed as a fraction of the relative abundance of 18S ribosomal RNA (rRNA) in arbitrary units to correct for variations in gel loading and efficiency of RNA transfer, as previously described (29, 30).

Probes

cDNAs encoding ovine GR (942-bp cDNA fragment) and ovine 11βHSD1 (1257-bp full-length cDNA; a gift from Dr. Kaiping Yang, The Lawson Research Institute, London, Canada) (31, 32) were subcloned into the *Eco*RI site of pBluescript KS (Stratagene, La Jolla, CA). The ovine 11βHSD2 cDNA (1838-bp full-length cDNA; donated by Prof. Perrin White, University of Texas Southwestern Medical Center, Dallas, TX) (33) was subcloned into pTF1. The partial-length cDNA encoding ovine AT1 was PCR cloned using primers complimentary to the bovine AT1 sequence and subcloned into the *Spe*I and *Kpn*I sites of pBluescript SK (Stratagene) as previously described (34). The partial-length cDNA encoding ovine AT2 was similarly PCR cloned using primers complimentary to the rat AT2 sequence and subcloned into the *Eco*RI and *Hin*dIII sites of the pT7/T3 transcription vector (Ambion, Inc., Austin, TX) as previously described (35). The full-length cDNA encoding rat 18S rRNA (provided by Dr. Ira Wool University of Chicago, Chicago, IL) (36) was subcloned into the *Eco*RI site of pBluescript KS (Stratagene).

cDNA probes 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) (29–32). For AT1 and AT2, antisense cRNA probes were synthesized by T7 RNA polymerase-directed transcription after linearization of their respective purified vectors with *Nde*I and *Hin*dIII, respectively (34, 35). The cRNA probes were radiolabeled by incorporation of [^{32}P]UTP (3000 Ci/mmol), and the synthesis of more than 90% full-length transcripts was confirmed by PAGE as previously described (29).

Assays of 11βHSD activities

11βHSD1-mediated 11β-dehydrogenase and 11-oxoreductase activities and 11βHSD2-mediated 11β-dehydrogenase activity were determined by radiometric conversion assays similar to those described by Yang and colleagues (37, 38). Briefly, triplicate aliquots of each tissue were homogenized in 5–10 vol ice-cold 250 mm sucrose/10 mm sodium phosphate buffer (pH 7.0) containing a protease inhibitor (1 mm phenylmethylsulfonylfluoride). 11β-Dehydrogenase activity was measured by determining the rate of conversion of cortisol to cortisone in triplicate assay tubes containing tissue homogenate (300–500 μg/ml protein, assayed by the Bradford method), unlabeled cortisol (50–1000 nm) spiked with tracer, *i.e.* [1,2-^{3}H]cortisol (51.5 Ci/mmol; NEN Life Science Products-DuPont, Germany), and also 1 mm cofactor (NAD for 11βHSD2 and NADP for 11βHSD1 activities) in a 100-mm sodium phosphate buffer (pH 7.5) at 37 C in a shaking water bath. In keeping with previous studies (37, 38), reaction rates were found to be linear between 20 and 90 min incubation across protein concentrations of 200–700 μg/ml, so all incubations were halted after 40 min by placing tubes on ice. Steroids were quantitatively extracted in ethyl acetate/diethyl ether (4:1) and chromatographically separated by spotting, along with standards, onto TLC plates that were subsequently developed using a mobile phase of chloroform/methanol (9:1) as previously described (37, 38). Steroids were eluted from areas of the TLC plate corresponding to cortisol and cortisone, and levels of radioactivity were counted in a liquid scintillation counter. The rate of reaction (picomoles of cortisone formed per mg protein/h) was calculated from percent conversions between 10–20%, which equated to the linear part of the reaction velocity *vs.* substrate concentration plot, *i.e.* enzyme activities were analyzed under conditions of first order kinetics.

Levels of 11-oxoreductase activity were measured using similar methodology, except that cortisone (50–1000 nm) was employed as substrate, [1,2-^{3}H]cortisone was used as tracer, NADH/NADPH (1 mm) was used as cofactor, and 100 mm sodium phosphate buffer (pH 6.0) was used as incubation buffer. Tritiated cortisone was prepared as previously described (39). For both 11β-dehydrogenase and 11-oxoreductase activities, the sum of cortisol and cortisone that was extracted from the tissue homogenates after incubation was not significantly different from that for the substrate that had been added before incubation. This confirmed the absence of measurable levels of metabolites of either cortisol or cortisone by other steroidogenic enzymes that may have been present in the tissues.

Statistics

All data were found to be normally distributed by Kolmogorov-Smirnov analysis and are presented as the mean ± sem. Mean fetal body weight, crown-rump length, organ weights, kidney dimensions, and expression of GR, 11βHSD1, 11βHSD2, AT1, and AT2 in each tissue were compared between NR and control offspring by Student's *t* test, assuming unequal variance. One-way ANOVA was employed to determine whether there was a significant effect of maternal nutrition on parameters pertaining to fetal outcomes, organ size, and gene expression. There was no effect of offspring gender on any measurement made, so results between sexes were pooled as there was also no difference in sex ratio distribution between groups (nutrient-restricted, 15 females and 14 males; controls, 10 females and 10 males). Statistical analyses were performed using SPSS software (SPSS, Inc., Chicago, IL), and $P < 0.05$ was considered significant. Analyses of imprecision for Northern blot analyses of mRNA expression revealed coefficients of variations within a gel of less than 9% and between gels of less than 14%. Similar analyses of imprecision for enzyme activity assays revealed coefficients of variation of less than 6%.

Results

Placental weight and neonatal organ size

Maternal nutrient restriction in early to midgestation resulted in unchanged offspring body weight at term, but significantly greater total placental weight ($P < 0.05$) and significantly higher term placental to neonatal weight ratio ($P < 0.01$; Table 1). Neonates born to NR ewes tended to have larger adrenal glands, liver, and lungs, but these differences did not reach statistical significance (Table 1). However, kidney weight was significantly greater in neonatal offspring delivered from NR ewes compared with controls (Table 1). The weights of other organs, including heart, hypothalamus, pituitary, and perirenal adipose tissue, were similar in the neonatal offspring from the two diet groups (data not

TABLE 1. Feto-placental and organ size of neonatal sheep offspring close to term from nutrient-restricted (NR) ewes (n = 29) and control-fed ewes (n = 20)

Growth parameter	Control	NR
BW (kg)	3619 ± 112	3895 ± 95
Crown-rump length (cm)	48.2 ± 0.6	50.4 ± 0.4[a]
Placental wt (g)	368.7 ± 24.5	413.0 ± 23.3[a]
Placental/neonate wt ratio	0.66 ± 0.03	0.72 ± 0.01[a]
Liver wt (g)	81.1 ± 5.7	96.1 ± 8.3
Lung wt (g)	77.8 ± 7.4	104.9 ± 14.7
Adrenal wt (g)	0.64 ± 0.42	0.86 ± 0.31
Kidney wt (g)	16.9 ± 2.3	20.8 ± 1.4[a]
Kidney length (mm)	37.0 ± 1.0	32.5 ± 1.5[b]
Kidney transverse width (mm)	18.3 ± 0.4	20.6 ± 0.5[c]
Kidney lateral width (mm)	23.0 ± 0.8	22.0 ± 0.4
Kidney length/wt	4.23 ± 0.14	3.46 ± 0.10[c]
Kidney transverse width/length	0.50 ± 0.01	0.61 ± 0.01[b]

Data are the mean ± SEM. Significant differences between groups are indicated.

[a] $P < 0.05$.

[b] $P < 0.001$.

[c] $P < 0.01$.

shown). In addition to kidney weight, kidney dimensions were significantly altered in the neonatal offspring from NR ewes. Thus, the length of kidneys from NR offspring was significantly reduced, and the transverse width was significantly increased compared with control values (Table 1).

GR mRNA expression in fetal and neonatal tissues

The abundance of GR mRNA expression was markedly greater in adrenals, liver, lungs, perirenal adipose tissue, whole kidney (Fig. 1), and renal cortex (NR *vs.* control, GR mRNA/18S rRNA, 0.58 ± 0.03 *vs.* 0.33 ± 0.02; $P < 0.001$) in neonatal offspring from NR ewes compared with controls. Conversely, maternal nutrient restriction during early to midgestation had no significant effect on GR mRNA expression in central tissues of these offspring, such as the hypothalamus (NR *vs.* control, GR mRNA/18S rRNA, 0.186 ± 0.012 *vs.* 0.137 ± 0.028; P = NS) and the pituitary (NR *vs.* control, GR mRNA/18S rRNA, 0.175 ± 0.029 *vs.* 0.221 ± 0.017; P = NS).

Elevated GR mRNA expression was also evident in peripheral tissues, such as liver and lung (Fig. 2), from fetal sheep at 77 days gestation, *i.e.* immediately after cessation of a 50-day period of maternal nutrient restriction. Taken together, these data indicate that the stimulatory effect of maternal nutrient restriction on GR expression in the adrenal gland and peripheral corticosteroid target tissues of the offspring was not a transient response to nutrient restriction, but persisted despite restoration of maternal nutrition from 77 days gestation to term.

11βHSD1 and 11βHSD2 mRNA expression in placenta and fetal and neonatal tissues

11βHSD1 mRNA expression was significantly greater in the maternal component of the ovine placenta compared with the fetal placenta at 144–146 days gestation (maternal *vs.* fetal, 11βHSD1 mRNA/18S rRNA, 0.51 ± 0.08 *vs.* 0.32 ± 0.09; $P < 0.01$). Abundance of 11βHSD1 mRNA was greater in term placental tissue from the NR ewes, but this did not reach significance with respect to either the maternal component (NR *vs.* control, 11βHSD1 mRNA/18S, 1.32 ± 0.15 *vs.* 0.82 ± 0.20; P = NS) or the fetal component (NR *vs.* control, 11βHSD1 mRNA/18S, 0.61 ± 0.11 *vs.* 0.46 ± 0.12; P = NS).

Early to midgestational maternal nutrient restriction had no effect on the expression of 11βHSD1 in the neonatal offspring central and peripheral tissues that were analyzed, with the exception of perirenal adipose tissue, in which levels were elevated by approximately 2-fold (Fig. 3). 11βHSD1 expression was undetectable in fetal offspring tissues on day 77 of gestation. There was close agreement between levels of 11βHSD1mRNA expression and enzyme activity (*e.g.* in neonatal liver and perirenal adipose tissue; $r^2 = 0.83$; $P < 0.001$; n = 30). Thus, in perirenal adipose tissue from the 49 neonatal offspring, although 11β-dehydrogenase activity was negligible, levels of 11-oxoreductase activity were significantly higher in the NR group compared with controls (NR *vs.* control, 104 ± 9 *vs.* 62 ± 6 pmol cortisol formed/mg protein·h; $P < 0.01$). These levels of enzyme activity are comparable to those described previously in fetal sheep tissues (37, 38).

Notwithstanding the stimulatory effects of maternal nutrient restriction on perirenal adipose tissue expression of 11βHSD1, the absence of any effect in all other tissues contrasted with the marked responses of 11βHSD2 expression. Thus, maternal undernutrition resulted in a 50% decline in the levels of 11βHSD2 mRNA expression in all neonatal offspring tissues in which this key enzyme was found to be abundant, *i.e.* in kidney and adrenal (Fig. 4).

11βHSD2 mRNA expression was undetectable in either maternal or fetal placental tissue near term. Conversely, abundant 11βHSD2 expression was evident in midgestation placental tissue. Maternal nutrient restriction resulted in a 50% decline in placental 11βHSD2 expression at 77 days gestation (Fig. 5A). 11βHSD2 expression was approximately 50% lower in other midgestational fetal tissues, *e.g.* the adrenal glands (Fig. 5B) from offspring from NR ewes compared with controls.

11βHSD2 mRNA was closely correlated with those for NAD-dependent 11β-dehydrogenase activity (*e.g.* in neonatal kidney; $r^2 = 0.88$; $P < 0.001$; n = 37), which this mRNA has been shown to encode (31). Thus, in the kidney from the 49 neonatal offspring, for example, levels of 11β-dehydrogenase activity, like those of 11βHSD2 mRNA, were significantly lower in the NR group compared with controls (NR *vs.* control, 172 ± 15 *vs.* 244 ± 13 pmol cortisone formed/mg protein·h; $P < 0.01$). These levels of renal 11β-dehydrogenase activity are similar to those reported by others in fetal and neonatal sheep kidney (37). In keeping with the persistent effects of previous maternal nutrient restriction on offspring tissue expression of the GR, attenuated levels of 11βHSD2 expression in neonatal adrenal and kidney persisted despite the restoration of maternal nutrition to approximately 100% of ME requirements during the second half of gestation.

AT1 and AT2 mRNA expression in fetal and neonatal tissues

Abundant AT1 mRNA expression was detected in the adrenal gland, liver, lung, and kidney of both male and

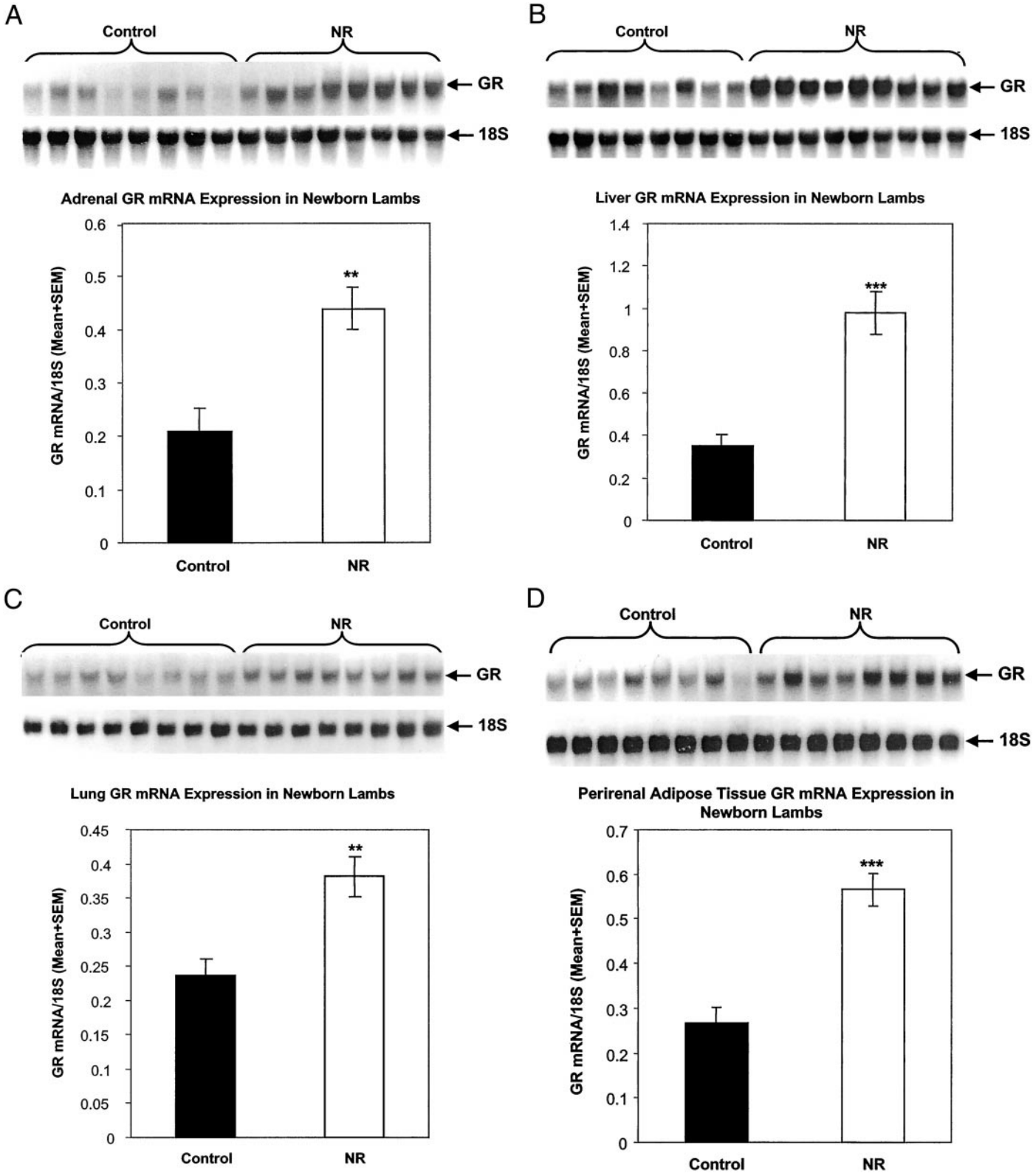

FIG. 1. *Top panel*, Northern blot analysis of GR mRNA (5.6 kb) expression in total RNA isolated from representative samples of adrenal glands (A), liver (B), lungs (C), perirenal adipose tissue (D), and whole kidney (E) of neonatal offspring born to NR or control-fed ewes. Even loading of the gel was confirmed by probing for 18S rRNA. *Bottom panel*, Histograms depicting mean group levels of GR mRNA relative to 18S rRNA in each of these tissues. Values are the mean ± SEM (NR, n = 29; controls, n = 20). Significant differences between groups are indicated (**, $P < 0.01$; ***, $P < 0.001$).

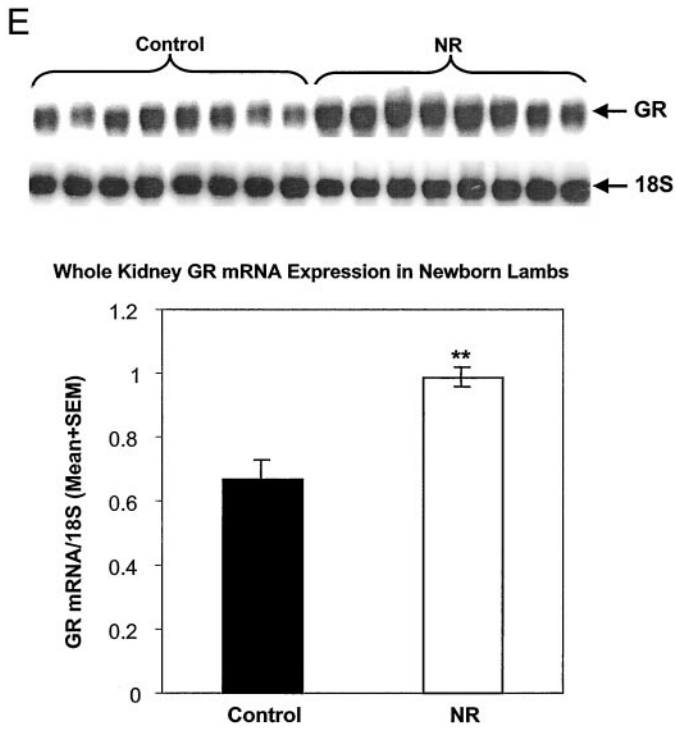

FIG. 1 Continued

female neonatal lambs (Fig. 6). Importantly, maternal nutrient restriction during early to midgestation resulted in markedly greater AT1 expression in these offspring tissues. AT1 mRNA was also abundantly expressed in the cardiac ventricles, but there was no effect of maternal nutrient restriction on its expression. For example, AT1 mRNA expression was similar in the left ventricles from NR offspring compared with controls (NR *vs.* control, AT1 mRNA/18S rRNA, 0.302 ± 0.015 *vs.* 0.315 ± 0.023; P = NS).

The marked stimulatory effect of early to midgestation maternal nutrient restriction on AT1 mRNA expression in adrenal, kidney, liver, and lung from the neonatal offspring was also evident in fetal tissues on day 77 of gestation, *i.e.* immediately after cessation of 50 days of undernutrition. For example, in a key angiotensin II target tissue, the adrenal gland, AT1 mRNA was approximately 1.7-fold higher in the NR offspring compared with the control value (NR *vs.* control, AT1 mRNA/18S rRNA, 0.632 ± 0.033 *vs.* 0.394 ± 0.037; $P < 0.01$). Thus, in keeping with the persistent effects of transient maternal nutrient restriction on offspring tissue expression of GR and 11βHSD2, not only did this brief period of nutrient restriction increase AT1 mRNA expression in immediate response to the undernutrition, but, importantly, this effect persisted despite restoration of 100% energy requirements during the second half of gestation. In contrast with the marked stimulatory effects of early to midgestational maternal nutrient restriction on expression of AT1 in the kidney and other tissues, AT2 mRNA expression was not affected by undernutrition in any of the tissues analyzed (data not shown).

Discussion

The present study has demonstrated, for the first time, that maternal nutrient restriction for a defined brief period during early to midgestation results in tissue-specific increases in the expression of GR, 11βHSD1, and AT1 and attenuated expression of 11βHSD2 in adrenal and kidney in neonatal sheep. The maintenance of these effects beyond the period of nutrient restriction despite restoration of maternal nutrition to requirements indicates that the expression of these genes has been programmed (*i.e.* results in persistent changes beyond the immediate period of nutrient restriction) by maternal nutritional status during a window of sensitivity in early pregnancy.

Maternal undernutrition between days 28 and 77 of ovine pregnancy programmed increases in GR mRNA expression in the adrenals, kidney, liver, lungs, and perirenal adipose tissue; increases in expression of 11βHSD1 mRNA in perirenal adipose tissue (but not in other tissues); and marked reductions in 11βHSD2 mRNA expression in the adrenal and kidney. This was accompanied by increased expression of an important component of the RAS, *i.e.* glucocorticoid-responsive AT1 (15, 16), only in those tissues in which increases in GR and/or decreases in 11βHSD2 gene expression were evident, *i.e.* adrenal, kidney, liver, and lung. Notwithstanding the absence of AT1 mRNA expression in perirenal adipose tissue, these data suggest tissue-specific programming of glucocorticoid hormone action and tissue sensitivity by maternal undernutrition. Importantly, these effects were observed in the absence of any major change in the maternal or fetal metabolic or hormonal environment (24, 25, 40).

Intrauterine programming of GR, 11βHSD1, 11βHSD2, and AT1 gene expression by nutrient restriction was not associated with impaired fetal growth (as defined by body weight) (25). There was no effect on neonatal organ size, except for the kidneys, which were approximately 25% heavier, increased in width, and shorter in length, similar to those in the hypertensive offspring of rats fed a low protein diet during pregnancy (28). The significantly smaller kidney length in relation to kidney weight in the neonatal offspring from NR ewes, and their significantly greater transverse widths in relation to length, indicate that the stimulatory effects of nutrient restriction favor a shorter, but broader, kidney. Previous nutrient restriction also promoted increased placental growth and offspring length near term. These outcomes are in keeping with those of maternal nutrient restriction in both humans and sheep (24–26).

In contrast, McMillen and co-workers have shown that fetal undernutrition induced by placental restriction, with chronic persistent fetal hypoxemia, hypoglycemia, and altered fetal stress hormone levels similar to those seen in severely growth-retarded human fetuses (41), results in impaired fetal growth, increased hepatic 11βHSD1 mRNA expression, and no effect on renal or adrenal 11βHSD2 mRNA expression during fetal life (30). This clearly represents a different, much more severe and prolonged, challenge to the fetus throughout gestation. However, intermittent fetal hypoxemia and nutrient deprivation induced by placental embolization decreases fetal renal 11βHSD2 expression (42) as in the present study. In addition, as gene expression was

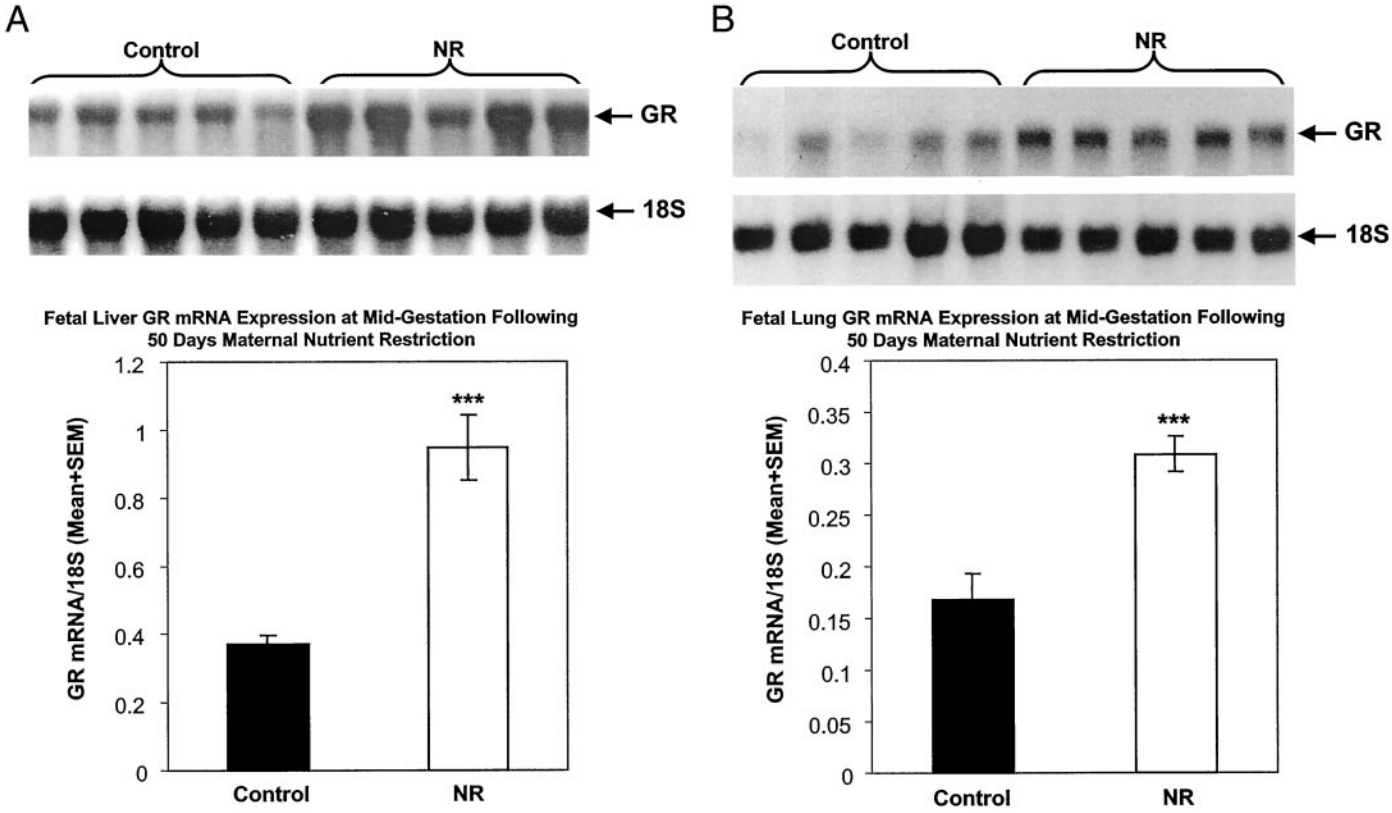

FIG. 2. *Top panel*, Northern blot analysis of GR mRNA (5.6 kb) expression in total RNA isolated from representative samples of liver (A) and lung (B) of fetuses sampled from NR ewes (n = 5) or control-fed ewes (n = 5) at 77 days gestation (term = 147 days). Even loading of the gel was confirmed by probing for 18S rRNA. *Bottom panel*, Histograms depicting mean group levels of GR mRNA relative to 18S rRNA in each of these tissues. Values are the mean ± SEM. Significant differences between groups are indicated (***, $P < 0.001$).

examined concurrently with the challenge to the fetus in these earlier studies (30, 42), this may reflect transient responses rather than programmed events.

The immense clinical significance of epidemiological data of the fetal origins of adult disease lies in the strong associations between adult risk of CVD and modest reductions in body weight or thinness at birth, arising from transient changes in maternal nutrition (2–4) and not from severe, or rarer, cases of intrauterine growth retardation. The major significance of our findings is highlighted by an increasing number of epidemiological studies that reveal that low birth weight *per se* is not necessarily an informative index, and one of the strongest neonatal predictors of these CVD risk factors is to have been born long and thin, but not growth retarded (4). This phenotype resembles that of the neonatal progeny of NR ewes (25). Maternal nutritional programming, without reduction in fetal weight at birth, can be mimicked in the sheep by treatment with synthetic glucocorticoid during early to midgestation (12). The offspring develop a hyperactive hypothalamic-pituitary-adrenal axis and raised blood pressure in later life (12, 23).

The potent effects of glucocorticoids on fetal growth, development (16), and blood pressure (17, 18) are well documented. Our data also indicate programming of increased sensitivity to glucocorticoid in tissues involved in glucose and lipid metabolism as well as catecholamine and corticosteroid synthesis, but not in tissues mediating central feedback control of the hypothalamic-pituitary-adrenal axis. This provides plausible molecular mechanisms by which increased hypothalamic-pituitary-adrenal axis activity and glucocorticoid hormone action could mediate the link between events during intrauterine life and features of the metabolic syndrome in adult life in human populations (21).

The absence of a programming effect of maternal nutrient restriction on 11βHSD1 gene expression in any of the neonatal sheep tissues examined, with the exception of perirenal adipose tissue, is in keeping with a recent report describing 11βHSD1 expression in a maternal glucocorticoid treatment model of programmed hypertension in the rat (11). Given the findings from the current study and the previously established close correlation between levels of 11βHSD1 mRNA and 11-oxoreductase activity (22, 38), this suggests that 11βHSD1 is unlikely to contribute significantly to mechanisms by which altered levels of glucocorticoid hormone action are programmed in these tissues. However, in perirenal adipose tissue programmed increases in the expression of 11βHSD1 and resulting 11-oxoreductase activity suggest that 11βHSD1 may further increase the sensitivity to glucocorticoid in this tissue beyond that which may be predicted from increased GR expression alone. Such depots of abdom-

inal fat have also been shown to express high levels of GR (43) and 11βHSD1 (22), which mediate glucocorticoid antagonism of insulin-induced lipogenesis and promote lipolysis (20). Programming of increased levels of 11βHSD1 and GR expression in central fat depots may mediate the role of glucocorticoids in the link between the intrauterine environment and hypertriglyeridemia in later life (21). Glucocorticoids also promote differentiation of preadipocytes into functional fat cells, in which 11βHSD1 plays a key regulatory role (22). Thus, programming of increased GR and 11βHSD1 expression in abdominal fat depots by NR may contribute to mechanisms linking gestational undernutrition and adult obesity in human populations (44).

In contrast, 11βHSD2 expression is predominantly confined to mineralocorticoid target tissues (*i.e.* kidney and distal colon) (22) and the adrenal gland (45, 46). Two groups have recently reported parallel increases in levels of 11βHSD2 mRNA expression and 11-dehydrogenase activity in fetal sheep kidneys throughout the latter half of gestation to term (30, 37). This may serve to protect the renal mineralocorticoid receptor from occupancy by the prepartum surge in fetal cortisol levels. The programmed decline in 11βHSD2 expression observed in the neonatal offspring from NR ewes is likely, therefore, to further enhance levels of both GR and, in the kidney, mineralocorticoid receptor-mediated action in these tissues by attenuating cortisol metabolism to inactive cortisone. In support of this, it has been shown that decreases in 11βHSD2 expression promote hypertension and increased expression of glucocorticoid- and mineralocorticoid-responsive genes in these tissues (22, 36, 46), which have potent effects on fluid-electrolyte homeostasis and vascular tone. This is consistent with the hypertensive effects of congenital or acquired 11βHSD2 deficiency in humans (22). The nutritional programming of attenuated 11βHSD2 expression in

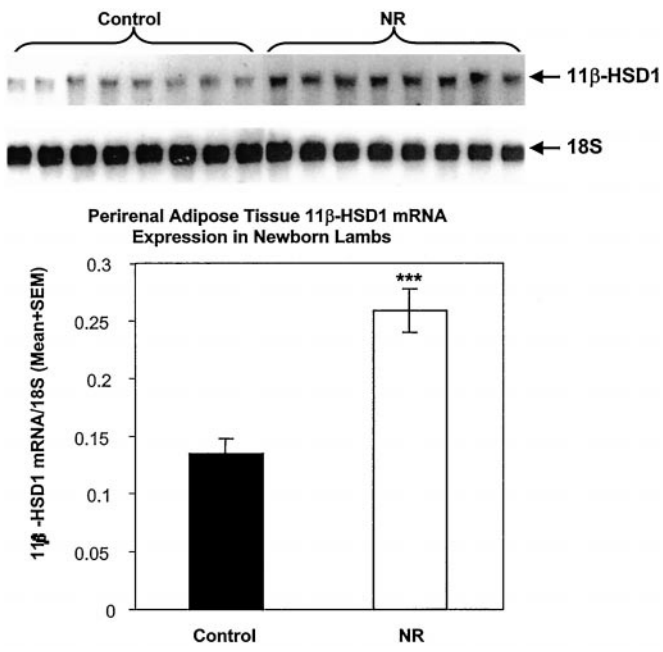

FIG. 3. *Top panel*, Northern blot analysis of 11βHSD1 mRNA (1.8 kb) expression in total RNA isolated from representative samples of perirenal adipose tissue of neonatal offspring born to NR or control-fed ewes. Even loading of the gel was confirmed by probing for 18S rRNA. *Bottom panel*, Histogram depicting mean group levels of 11βHSD1 mRNA relative to 18S rRNA. Values are the mean ± SEM (NR, n = 29; controls n = 20). Significant differences between groups are indicated (***, $P < 0.001$).

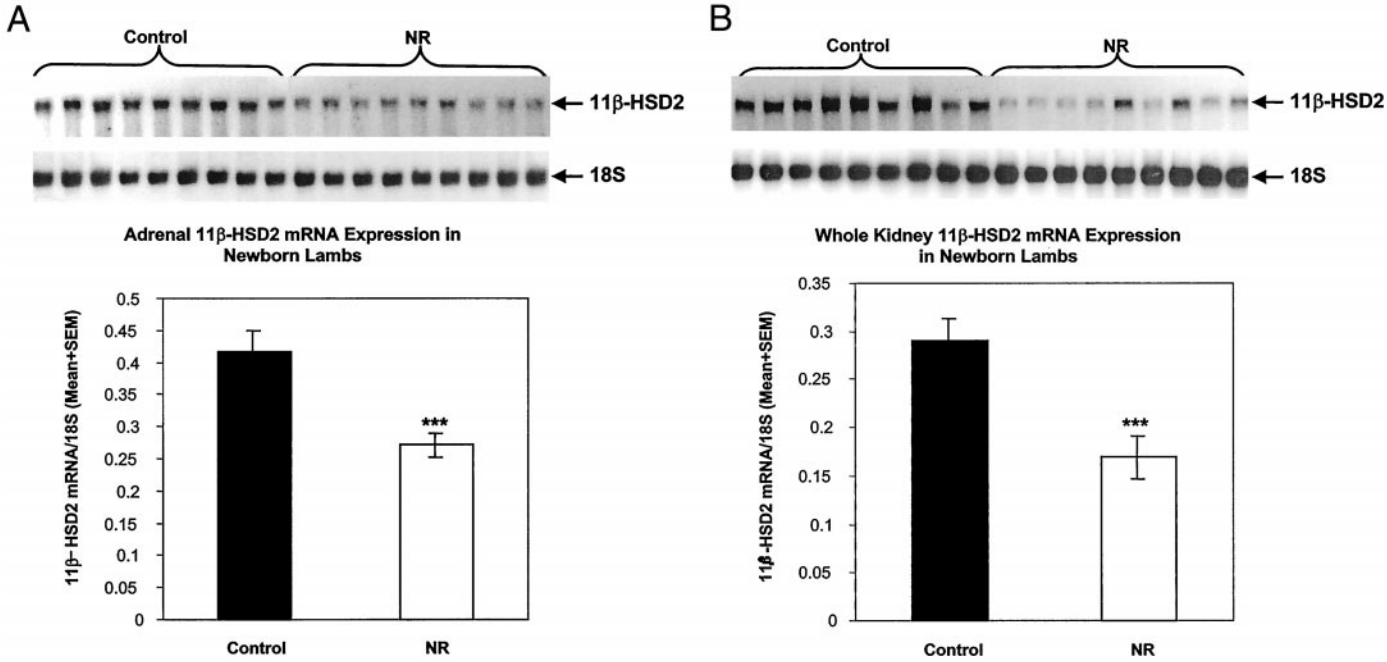

FIG. 4. *Top panel*, Northern blot analysis of 11βHSD2 mRNA (1.9 kb) expression in total RNA isolated from representative samples of adrenal (A) and whole kidney (B) of neonatal offspring born to NR or control-fed ewes. Even loading of the gel was confirmed by probing for 18S rRNA. *Bottom panel*, Histograms depicting mean group levels of 11βHSD2 mRNA relative to 18S rRNA in each of these tissues. Values are the mean ± SEM (NR, n = 29; controls n = 20). Significant differences between groups are indicated (***, $P < 0.001$).

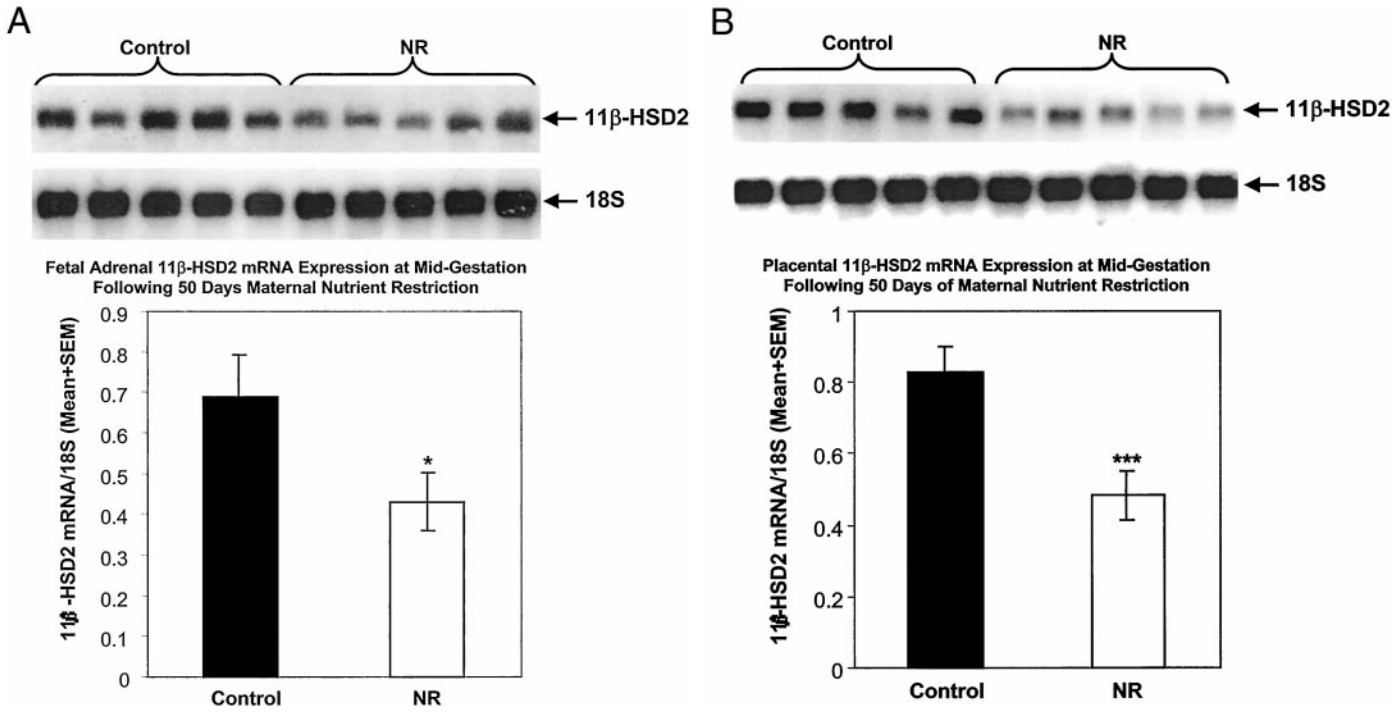

FIG. 5. *Top panels*, Northern blot analysis of 11βHSD2 mRNA (1.9 kb) expression in total RNA isolated from samples of placenta (A) and fetal adrenal (B) sampled from NR ewes (n = 5) or control-fed ewes (n = 5) at 77 days gestation (term = 147 days). Even loading of the gel was confirmed by probing for 18S rRNA. *Bottom panels*, Histograms depicting mean group levels of 11βHSD2 mRNA relative to 18S rRNA in each of these tissues. Values are the mean ± SEM. Significant differences between groups are indicated (***, $P < 0.001$).

the present study and in the maternal low protein rat model (7) may represent a fundamental mechanism linking fetal development with hypertension in later life.

In keeping with the recently described ontogeny of renal 11βHSD2 mRNA expression during the third trimester of ovine gestation (30), AT1 mRNA expression has been shown to increase toward term (34, 35), concurrent with the progressive rise in fetal circulating levels of cortisol and consistent with glucocorticoid induction of AT1 expression (16, 17). Paradoxically, Robillard and co-workers have shown that a cortisol infusion during late gestation can both up- and down-regulate AT1 expression in a tissue-specific manner (35).The additional major finding of the present study is that glucocorticoid-responsive AT1 gene expression in neonatal offspring from NR ewes was only elevated in those tissues in which GR expression was markedly increased and/or 11βHSD2 expression was substantially decreased. Indeed, we observed no effect on AT1 expression in offspring heart ventricles, in which glucocorticoids have been shown to up-regulate AT1 expression (35), but in which maternal nutrient restriction had no effect on GR expression or ventricular size. This is consistent with the hypothesis that tissue-specific programming of changes in GR and 11βHSD2 expression promotes increased levels of glucocorticoid action in these tissues.

AT1 expression is a key component of the RAS control of blood pressure and cardiovascular state during both fetal and postnatal life (18). Thus, AT1 mediates the vasoactive and other functions of angiotensin II in the regulation of fluid-electrolyte transport, smooth muscle contraction, adrenal aldosterone synthesis, and cell growth (18). Given the close correlation between levels of AT1 mRNA and the biological potency of angiotensin II (17), the increased levels of AT1 mRNA expression in the offspring of NR ewes may have mediated the effects of maternal undernutrition on kidney size and morphology and the trend toward increased adrenal, liver, and lung weight described in the present study. Recent functional studies in the maternal low protein rat model suggest that programming of increased AT1 expression is an important causal mechanism by which maternal undernutrition during pregnancy results in raised offspring blood pressure (47). Our findings implicate molecular mechanisms underlying the key potential role for the RAS in the prenatal programming of hypertension.

In summary, we have demonstrated that a relatively brief period of maternal undernutrition during early to midgestation exerts marked tissue-specific effects on the expression of GR, 11βHSD1, 11βHSD2, and AT1. This is associated with increased placental size and longer, thinner (but not low birth weight) offspring in which the effects of earlier maternal nutrient restriction on the expression of these genes have persisted. Thus, these offspring exhibit prenatally programmed increases in peripheral, but not central, tissue sensitivity to glucocorticoid that are evident at term by tissue-specific increases in the expression of GR, 11βHSD1, and AT1 and decreased expression of 11βHSD2. These data suggest important potential molecular mechanisms by which maternal nutritional manipulations of this nature result in the program-

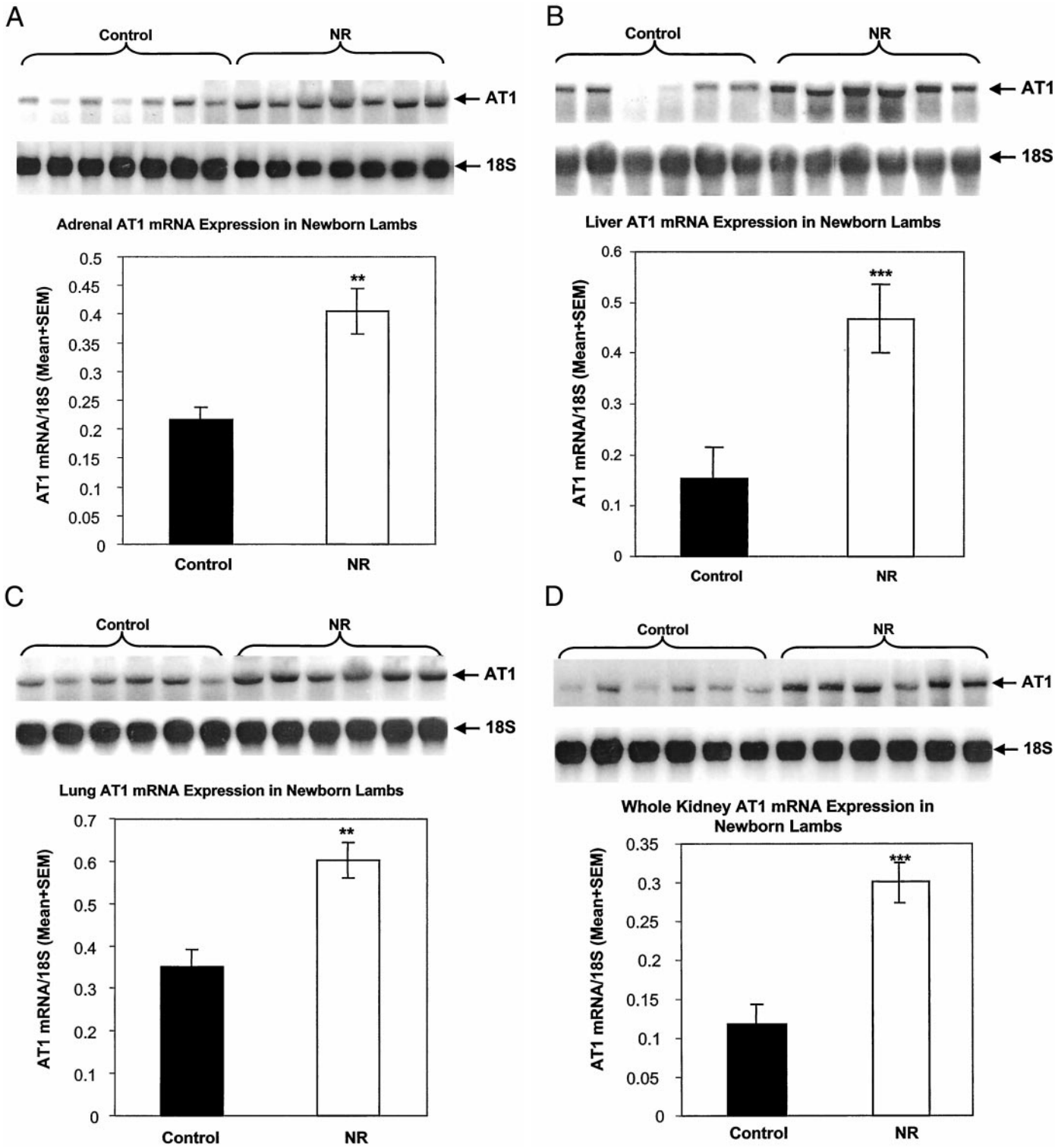

FIG. 6. *Top panels*, Northern blot analysis of AT1 mRNA (~3.0 kb) expression in total RNA isolated from representative samples of adrenal glands (A), liver (B), lung (C), and whole kidney (D) of neonatal offspring born to NR or control-fed ewes. Even loading of the gel was confirmed by probing for 18S rRNA. *Bottom panels*, Histograms depicting mean group levels of AT1 mRNA relative to 18S rRNA in each of these tissues. Values are the mean ± SEM (NR, n = 29; controls n = 20). Significant differences between groups are indicated (**, $P < 0.01$; ***, $P < 0.001$).

ming of raised blood pressure. They also raise the possibility that programmed tissue-specific changes in the expression of these genes may underlie the molecular mechanisms by which similar maternal undernutrition during pregnancy increases offspring risk of hypertension, insulin resistance, and CVD in human populations.

Acknowledgments

The authors express their gratitude to Mrs. Carol Porter for her technical assistance.

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