

Protein restriction during pregnancy induces hypertension in adult female rat offspring – influence of oestradiol

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Abstract

We previously reported that gestational dietary protein restriction in rats causes sex-related differences in development of blood pressure (BP) in the offspring, which is more pronounced in males than in females. As such effects may depend on sex hormones, we investigated the role of oestradiol in the development of hypertension in female offspring of protein-restricted dams. Female offspring of pregnant rats fed normal (20%) or protein-restricted (6%) casein diets throughout pregnancy were kept either intact, ovariectomised or ovariectomised with oestradiol supplementation. BP, Plasma oestradiol and testosterone levels, and vascular oestrogen receptor (ER) were examined. BP was significantly higher and plasma oestradiol levels were significantly lower (–34%) in intact protein-restricted female offspring compared to corresponding controls. Further decrease in oestradiol levels by ovariectomy exacerbated hypertension in the protein-restricted females, with an earlier onset and more prominent elevation in BP compared to controls. Oestradiol supplementation in ovariectomised protein-restricted females significantly reversed ovariectomy-induced hypertension but did not normalise BP to control levels. The hypertensive protein-restricted females have reduced vascular ER α expression that was unaffected by ovariectomy or oestradiol replacement. In addition, testosterone levels were significantly higher by 2.4-, 3.4- and 2.8-fold in intact, ovariectomised and oestradiol-replaced protein-restricted females compared to corresponding controls. The present data show that: (1) hypertension in protein-restricted adult female offspring is associated with reduced plasma oestradiol levels; (2) oestradiol protects and limits the severity of hypertension in protein-restricted females and contributes to sexual dimorphism; (3) oestradiol replacement fails to completely reverse hypertension, which may be related to limited availability of vascular ER α receptors and/or increased circulating testosterone levels.

Key words: Blood pressure: Sex steroid hormones: Sex differences: Fetal programming: Oestradiol: Sprague–Dawley rats

Epidemiological evidence suggests that adverse fetal environment contributes to the development of adult health disorders^(1,2). Animal studies have shown that nutrient, energy or oxygen restriction, or reduction in placental perfusion during pregnancy often results in fetal growth restriction and hypertension during adult life^(3–5), although the mechanisms are still not completely understood. In the rat, we and others^(6–8) have demonstrated that protein restriction during pregnancy results in fetal growth restriction and hypertension in adult offspring. Alterations in function of vascular smooth muscle, endothelium, renin–angiotensin system and hypothalamic–pituitary–adrenal axis are thought to participate in both the development and maintenance of prenatally programmed hypertension^(9,10).

Studies examining the underlying mechanisms that contribute to hypertension of developmental origin should also take into account the well-established sex differences in the occurrence of CVD in humans at various stages of life.

The incidence of CVD and hypertension is lower in premenopausal women compared with age-matched men and postmenopausal women⁽¹¹⁾. However, after menopause, the risk of hypertension increases with age⁽¹¹⁾, suggesting that the fully functional ovaries reduce the risk for hypertension and CVD. In animal models of programming, sex differences are also apparent, with males most often affected more severely. In the rat, moderate maternal dietary protein restriction (10 v. 20% in controls) during pregnancy results in hypertension in adult male offspring, but not in females, and it is only when the insult becomes more severe that the female develops a hypertensive phenotype^(12,13). We have previously reported that severe protein restriction (6% protein diet compared to 20% in controls) during pregnancy causes hypertension in both male and female offspring, but the effect is more pronounced in male offspring^(6,8). These studies suggest that sex hormones may play an important role in modulating cardiovascular responses to an adverse fetal environment.

Abbreviations: BP, blood pressure; ER, oestrogen receptor; MAP, mean arterial pressure.

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Sex steroids – oestrogens in particular – are known to have cardiovascular protective effects^(14–16). Oestrogen deprivation by ovariectomy has been shown to exacerbate existing hypertension in female rats in some experimental models of hypertension^(15,17). We and others have previously shown that adult female offspring of protein-restricted dams have reduced plasma oestradiol levels^(6,18). On the basis of these findings, we hypothesise that reduced oestradiol levels may contribute to moderate increases in blood pressure (BP) in protein-restricted females, and further reduction in oestradiol levels by ovariectomy would cause elevation in BP to develop more rapidly postnatally. To establish the protective role of oestrogen, it is also important to examine whether oestradiol replacement reverses hypertension in protein-restricted females. Thus, the purpose of this study is to determine whether oestrogens protect against increases in BP in adult protein-restricted females.

Materials and methods

Animals and experimental protocols

All procedures were approved by the Animal Care and Use Committee at the University of Texas Medical Branch and were in accordance with those published by the US National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication no. 85–23, revised 1996).

Virgin female Sprague–Dawley rats (Harlan Sprague Dawley, Houston, TX, USA) aged 9–12 weeks were maintained at 23°C, 12 h light–12 h dark cycles, with free access to water and standard rat chow from Harlan Teklad (Madison, WI, USA). Female rats were mated with male Sprague–Dawley

rats, and conception was confirmed by the observation of sperm in vaginal smear. On day 1 of pregnancy (gestational length 22 d), rats were randomly divided into two groups of eight pregnant rats each and were fed either a control (20% casein; Harlan Teklad, catalogue no. TD.91352) or an isoenergetic protein-restricted (6% casein; Harlan Teklad, catalogue no. TD.90016) rat diet throughout pregnancy, as in our previous studies^(6,8). The pregnant rats were allowed to deliver at term, and after the delivery of pups, all lactating mothers (including protein-restricted dams) were maintained on standard rat chow. The pups were sexed based on anogenital distances, and litter sizes were adjusted to ten pups per dam, with equal proportion of males and females. Pups were sexed and weaned from their dams at 21 d of age, and only female offspring were used for the study. The rat offspring were fed with standard rat chow. As shown in Fig. 1, before puberty, at 7 weeks of age, offspring of both control and protein-restricted dams were divided into three groups: sham surgery (*n* 8 in each group), ovariectomy (*n* 8 each in each group) and ovariectomy with oestradiol replacement (control *n* 6; protein restricted *n* 8). In these groups of animals, changes in mean BP (non-invasive) were measured at 8, 12 and 16 weeks of age. At the end of 16 weeks, changes in mean BP were confirmed by measurement of mean arterial pressure (MAP) using a carotid arterial catheter. Following measurement of MAP, the animals were killed by CO₂ inhalation and blood was collected by cardiac puncture for the measurement of plasma oestradiol and testosterone levels. The aorta was harvested to determine the expression of oestrogen receptors (ER α and ER β). Care was taken not to take more than one sample per litter.

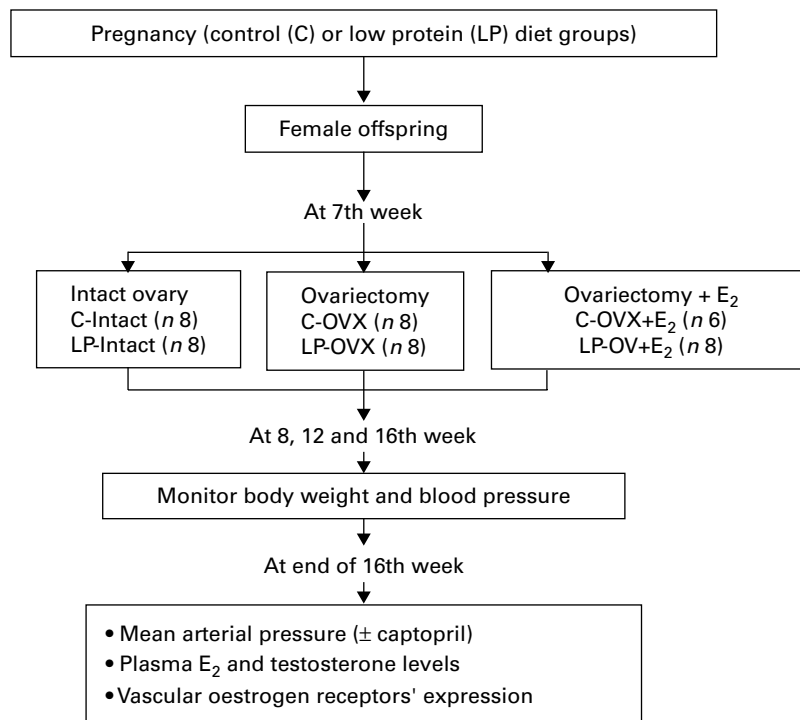


Fig. 1. Study design. OVX, ovariectomised; E₂, oestradiol.

Ovariectomy and oestrogen supplementation

Rats underwent ovariectomy, as described previously⁽¹⁹⁾, at 7 weeks of age. It is generally considered that in rats, puberty lasts until 54 d of age and then they are considered as adult, mature animals⁽²⁰⁾. In those rats receiving oestradiol supplementation, 17- β -oestradiol (5 μ g/kg in 100 μ l sesame oil; Sigma, St Louis, MO, USA) was injected subcutaneously once in every 4 d, immediately following ovariectomy and up to 16 weeks of age. Rats have an oestrous cycle length of 4 d; hence, this dosage regimen of oestradiol was chosen to closely mimic the physiological changes in 17- β -oestradiol levels across the rat oestrous cycle, as described previously^(21,22).

Non-invasive blood pressure measurement

Non-invasive BP was measured using a CODA computerised non-invasive BP system (Kent Scientific, Litchfield, CT, USA), as described previously⁽²³⁾. Rats were acclimatised for a week to the measurement procedures before testing. Rats were held in a preheated restrainer with the tail exposed, and both an occlusion cuff and a volume pressure-recording cuff were placed close to the base of the tail. The cuff was then inflated and deflated automatically within 90 s. BP is measured during thirty consecutive, computer-automated inflation/deflation cycles of the balloon cuff (ten preliminary measurements and twenty test measurements). Unlike other tail cuff systems, CODA uses volume pressure recording to measure both systolic and diastolic BP, which is then used by the software to calculate the mean BP. Data from the preliminary measurements are discarded and data from the test measurements are averaged. Signals were recorded and analysed using Kent Scientific software. To minimise stress-induced variations in BP, all measurements were taken by the same person in the same peaceful environment and at the same time of the day.

Mean arterial pressure

The MAP in various treatment groups was measured using an indwelling carotid arterial catheter in conscious, free-moving rats, as previously described⁽⁸⁾. Briefly, rats were anaesthetised (ketamine (45 mg/kg), xylazine (5 mg/kg); Burns Veterinary Supply, Westbury, NY, USA) and the carotid artery was cannulated with polyethylene tubing (PE-50; Becton Dickinson, Sparks, MD, USA). After a 1 d recovery period, the carotid cannula filled with heparinised saline (500 U/ml; Sigma) was connected to a pressure transducer to record MAP using the DBP001 direct BP system and Workbench for Windows software (Kent Scientific). The MAP was monitored continuously for 30 min to determine the baseline MAP.

Plasma oestradiol and testosterone levels

Blood samples were collected between 09.00 and 10.00 hours in all rats. In rats with intact ovaries the blood sample was collected on the day of oestrus, as determined by vaginal

cytology. In oestradiol supplemented rats, blood was collected 1 h after oestradiol supplementation. Plasma was separated by centrifugation and stored at -20°C until the time of measurement. Oestradiol and testosterone levels in the samples were measured using a RIA kit (Diagnostic Systems Laboratories, Inc., Webster, TX, USA) according to the manufacturer's instructions. The minimum detectable concentration of oestradiol was 5 pg/ml, whereas that of testosterone was 6 pg/ml. The intra- and interassay CV for oestradiol and testosterone were lower than 5%.

Western blot analysis

The aortas were homogenised in a solution containing 50 mM-Tris-HCl (pH 7.4), 2 mM-ethylene glycol tetra-acetic acid, 2 mM- β -mercaptoethanol and 1 mM-phenylmethylsulfonyl fluoride along with complete protease inhibitors (Roche, Indianapolis, IN, USA). Homogenates were centrifuged at 12 000 **g** for 5 min at 4°C , and the supernatant was used for determination of protein concentration using a bicinchoninic acid protein assay kit (Pierce, Inc., Rockford, IL, USA). Equal amounts of protein (20 μ g) were electrophoresed on 8% SDS-polyacrylamide gel and transferred onto nitrocellulose membrane. Membranes were blocked in PBS solution containing 5% dry milk for 1 h at room temperature. After blocking, membranes were probed with either monoclonal anti-ER α (1:1000; Upstate Biotechnology, Lake Placid, NY, USA) or polyclonal anti-ER β (1:1000; Affinity Bioreagents, Rockford, IL, USA), with overnight incubation in a Tris-buffered saline solution containing 5% milk. Membranes were washed using Tris-buffered saline containing 1% milk and then incubated with horseradish peroxidase-conjugated secondary antibody (dilution 1:2000; Cell Signaling, Danvers, MA, USA) for 1 h. Immunoreactive bands were visualised by enhanced chemiluminescence (Pierce Biotechnology). Developed films were scanned and analysed using Fluorchem (Alpha Innotech, San Leandro, CA, USA). The results were normalised with β -actin (1:5000; Sigma). β -Actin protein as a loading control did not differ among the groups.

Statistical analysis

Data were analysed using GraphPad Prism for Windows (GraphPad Software, San Diego, CA, USA). Two-way ANOVA followed by the Bonferroni *post hoc* tests were used for comparisons made between groups. For two-group comparisons, Student's *t* test was used. A value of $P < 0.05$ was considered statistically significant.

Results

Animal data

There were no differences in feed intake between control and protein-restricted dams during the gestation period. There were no significant differences in the mean litter size between control (14.4 (SEM 1.6)) and protein-restricted (13.8 (SEM 1.4)) groups. The birth weight (female only) was significantly lower

in the protein-restricted offspring (5.0 (SEM 0.1) g) compared to controls (6.1 (SEM 0.1) g). This observation of low birth weight in protein-restricted offspring is consistent with our previous publications^(6,8).

Mean blood pressure

We examined mean BP at 8, 12 and 16 weeks of age. In the intact protein-restricted offspring, the increases in mean BP were significant at 16 weeks of age, but not at 8 and 12 weeks of age compared to their corresponding controls (Fig. 2). Following ovariectomy in protein-restricted female offspring, there was an early onset of hypertension with more pronounced elevation in BP such that the BP was significantly higher at 12 and 16 weeks of age compared to the corresponding controls (Fig. 2). Thus, ovariectomy exacerbated mean BP in protein-restricted females without any effects in controls. Oestradiol replacement partially buffered the ovariectomy-induced increases in mean BP in protein-restricted females. However, the mean BP was significantly higher in the oestradiol-replaced, ovariectomised, protein-restricted females compared to the corresponding controls at 16 weeks of age.

Mean arterial pressure

To confirm the changes in mean BP obtained by a non-invasive system, we used direct measurement of MAP using indwelling carotid arterial catheters. MAP measured at 16 weeks of age was significantly higher in protein-restricted intact females compared to controls (120 (SEM 2) *v.* 101 (SEM 3) mmHg; Fig. 3). Ovariectomy further increased MAP in protein-restricted females (134 (SEM 5) mmHg), but was without significant effect in controls (104 (SEM 4) mmHg; Fig. 3). Thus, the MAP in protein-restricted ovariectomised females was significantly higher than that in ovariectomised

controls. Oestradiol replacement in ovariectomised females significantly reduced MAP in protein-restricted females (122 (SEM 3) mmHg), but was without significant effect in controls (98 (SEM 2) mmHg; Fig. 3). However, the mean BP in protein-restricted oestradiol-replaced females was significantly higher than that in their corresponding controls (Fig. 3). Thus, oestradiol replacement reduced ovariectomy-induced increases in mean BP, but did not normalise BP to control levels.

Plasma oestradiol and testosterone levels

Plasma oestradiol levels were significantly lower in protein-restricted intact females (−34%) compared to intact controls (Fig. 4). Ovariectomy significantly decreased oestradiol levels in controls and protein-restricted offspring compared to their respective intact offspring. There were no significant differences in oestradiol levels between protein-restricted ovariectomised females and their corresponding controls (Fig. 4). Oestradiol replacement in ovariectomised rats reinstated oestradiol levels in both control and protein-restricted females to levels comparable with that in controls with intact ovaries (Fig. 4).

Plasma testosterone levels were significantly higher in the protein-restricted intact females (2.4-fold) compared to corresponding controls (Fig. 4). Ovariectomy significantly decreased testosterone levels in controls, but not in protein-restricted females compared to their respective intact offspring; however, testosterone levels were significantly higher (3.4-fold) in protein-restricted ovariectomised females compared to respective controls (Fig. 4). Oestradiol replacement in ovariectomised rats did not significantly affect the testosterone levels in both control and protein-restricted females compared to their respective ovariectomised offspring; however, testosterone levels were significantly higher in oestradiol-replaced protein-restricted females (2.8-fold) compared to their corresponding controls (Fig. 4).

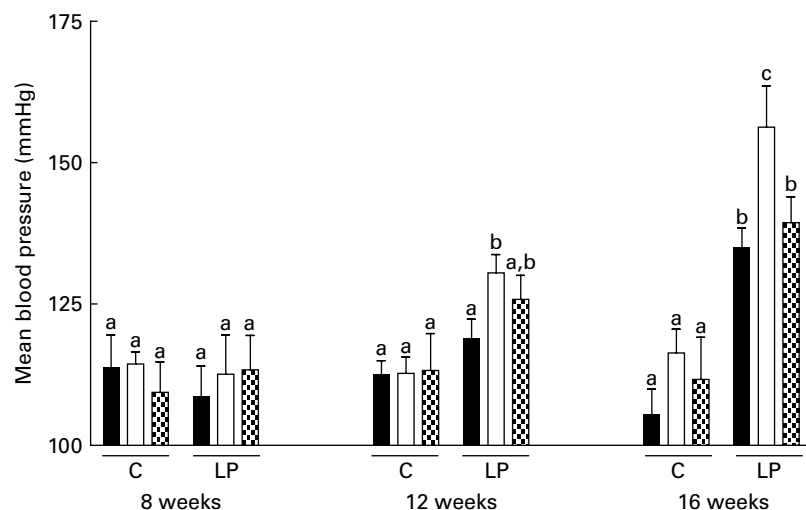


Fig. 2. Progressive changes in blood pressure in control (C) and protein-restricted female offspring at 8, 12 and 16 weeks of age. Changes in blood pressure were measured in intact (■), ovariectomised (OVX, □) and oestradiol (E₂) replaced (OVX + E₂, ▣) offspring using a non-invasive CODA system. ^{a,b,c}Mean values with unlike letters were significantly different ($P < 0.05$). Values are means with their standard errors for six to nine rats in each group. LP, low protein diet.

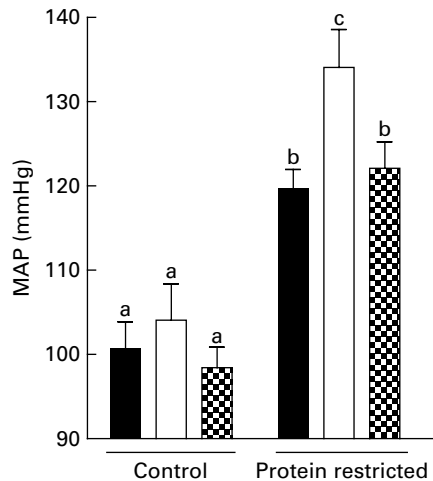


Fig. 3. Mean arterial pressure (MAP) in adult female control and protein-restricted offspring at 16 weeks of age. MAP was measured in conscious rats using indwelling carotid arterial catheters in intact (■), ovariectomised (OVX, □) and oestradiol (E_2) replaced (OVX + E_2 , ▨) offspring. ^{a,b,c} Mean values with unlike letters were significantly different ($P < 0.05$). Values are means with their standard errors for six to nine rats in each group.

Vascular oestrogen receptors

The expression of ER α protein in the aorta was significantly lower in protein-restricted intact females (-43%) compared to corresponding controls (Fig. 5). Ovariectomy decreased the ER α levels in controls, but was without significant effect in protein-restricted females compared to their respective intact offspring (Fig. 5). Thus, the vascular ER α protein in ovariectomised control and protein-restricted females was at a similar level. Oestradiol replacement reversed the ovariectomy-induced decrease in ER α levels in controls, but was without significant effect in protein-restricted females compared to their respective ovariectomised offspring (Fig. 5). Thus, the ER α levels were significantly lower in oestradiol-replaced protein-restricted females (-34%) compared to their corresponding controls. There were no changes in ER β levels among any control and low protein treatment groups (Fig. 5). Overall, it appears that protein restriction *in utero* programs for reduced expression of vascular ER α protein levels that may be less amenable to changes in circulating oestradiol levels.

Discussion

Maternal protein restriction programs the development of hypertension in the adult offspring^(5,6,8,13,24). We have reported previously that hypertension in the offspring subjected to severe protein restriction *in utero* is more pronounced in the males than in the females^(6,8). This study shows that hypertensive adult protein-restricted females have lower oestradiol levels and ovariectomy exacerbates the hypertension, suggesting that oestradiol plays a role in limiting the severity of hypertension and contributes to sexual dimorphism. Importantly, this study also demonstrates that oestradiol supplementation can only partially buffer BP,

probably due to a limited availability of vascular ER α receptors and/or increased circulating testosterone levels.

It is now well established that adverse fetal environments have long-term influences on the adult life BP. This study shows that the postpubertal adult female offspring of protein-restricted dams develop hypertension after 12 weeks of age. These findings are consistent with our previous reports^(6,8). In contrast to the present findings, Langley–Evans and colleagues reported that Wistar female offspring of modestly protein-restricted mothers developed hypertension at as early as 4 weeks of age⁽⁷⁾. The reason for this discrepancy is not clear. They used the Wistar strain, whereas we used Sprague–Dawley rats, although it seems unlikely that strain differences alone can account for the presence or absence of sex differences⁽²⁵⁾. In addition to the difference in the degree of protein restriction of Langley–Evans *et al.* and the present study, the other components of the diets used were not identical. However, consistent with the present findings, other investigators have reported that restriction of protein intake during pregnancy in rats lead to development of hypertension only after 75 d of age⁽²⁵⁾. It is possible that the protein-restricted prepubertal females are hypertensive and this effect is stabilised with puberty, as reported in the female offspring of nutrition-restricted dams⁽⁴⁾.

In addition, we show that these hypertensive adult protein-restricted females have lower plasma oestradiol levels compared to control females. As oestradiol is known to be

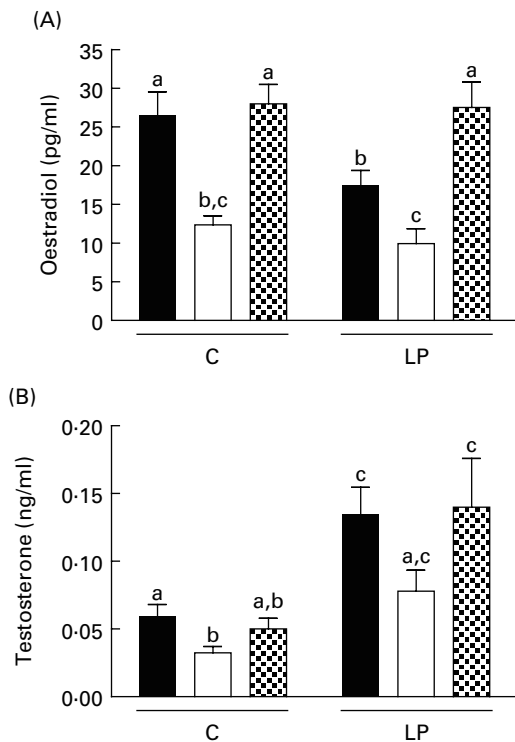


Fig. 4. Hormone levels in control (C) and protein-restricted offspring at 16 weeks of age. (A) Oestradiol (E_2) and (B) testosterone were measured by RIA in intact (■), ovariectomised (OVX, □) and E_2 replaced (OVX + E_2 , ▨) offspring. ^{a,b,c} Mean values with unlike letters were significantly different ($P < 0.05$). Values are means with their standard errors for six to nine rats in each group. LP, low protein diet.

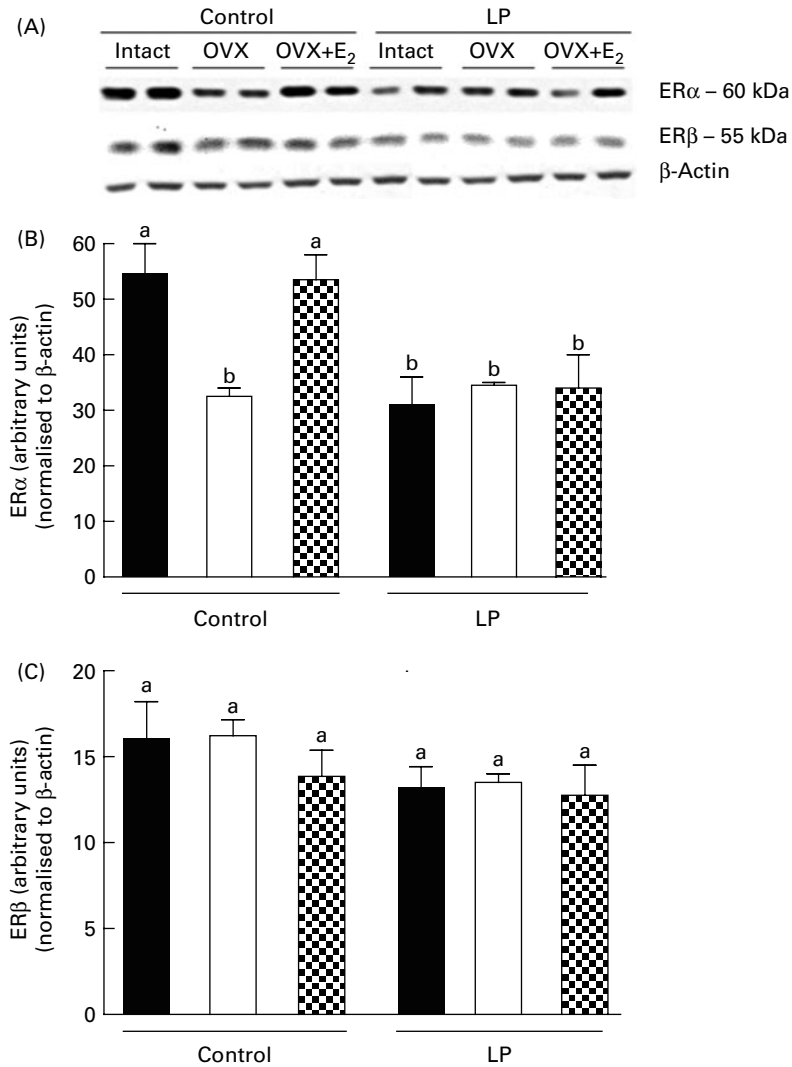


Fig. 5. (A) Blots of oestrogen receptor (ER) α and ER β proteins and β -actin. The summary of densitometry results of five to six animals in each group. Vascular protein levels of (B) ER α and (C) ER β receptors in intact (■), ovariectomised (OVX, □) and oestradiol (E₂) replaced (OVX + E₂, ▣) control and protein-restricted offspring at 16 weeks of age. Results were normalised against β -actin and expressed in arbitrary units. ^{a,b} Mean values with unlike letters were significantly different ($P < 0.05$). Values are means with their standard errors. LP, low protein diet.

vasoprotective^(14–16), we suggest that suboptimal oestradiol levels may allow for the moderate increases in BP. Compromised ovarian function may contribute to the reduction in oestradiol levels in protein-restricted females, as suggested by several reports; protein restriction *in utero* leads to delayed puberty⁽²⁶⁾, reduced ovarian size with decreased number of antral follicles and increased atretic follicles⁽²⁶⁾, impaired oestrus cyclicity^(8,27) and reduced fertility⁽²⁷⁾. The decrease in oestradiol concentrations may be consequent to decreases in aromatase enzyme activity reported in female offspring of protein-restricted dams^(28–30) (this is substantiated by the increase in testosterone levels observed in the gonad-intact protein-restricted offspring in this study). In addition, low levels of follicle-stimulating hormone, luteinising hormone⁽²⁷⁾ and their receptors⁽²⁸⁾ are observed in protein-restricted female offspring. Some of these mechanisms may underlie the reductions in oestradiol concentrations in protein-restricted animals.

We further show that ovariectomy decreased serum oestradiol to lower levels, consistent with published reports of oestradiol (11.2 (SEM 2.1) pg/ml) in ovariectomised rats⁽³¹⁾ (which are probably attributable to non-ovarian sources), exacerbating the hypertension with an earlier onset and more prominent elevation of BP in the protein-restricted females, which is comparable to that observed in adult male littermates⁽⁶⁾. This suggests that oestrogen indeed protects and limits the severity of hypertension in the present model and contributes to sex differences in the development of hypertension. Interestingly, the normotensive adult females that are born to pregnant rats with modest nutritional restriction have normal oestradiol levels and on ovariectomy they become hypertensive^(4,27), suggesting that optimal oestradiol levels in the intact animals could have conferred a cardiovascular protective effect.

To clarify the importance of oestradiol on BP regulation in protein-restricted female offspring, oestradiol replacement studies were initiated. Oestradiol supplementation to

protein-restricted ovariectomised female offspring only partially buffered the elevated BP. In addition, oestradiol replacement in intact offspring of protein-restricted dams, given at a dose that restores plasma levels to that observed in intact control offspring⁽³²⁾, did not return BP back to that seen in intact control offspring (116 (SEM 3.2) compared to 100 (SEM 3.1) mmHg in controls). Thus, it appears that oestradiol supplementation reversed only the ovariectomy-induced increase in BP but not that induced by maternal protein restriction. This finding is somewhat surprising, as it is in contrast to the findings in other rat models. In one model of genetically programmed hypertension, i.e. the mRen2.Lewis rat, it was shown that ovariectomy augments the BP of female animals and oestrogen supplementation protects against this process⁽¹⁷⁾. Ovariectomy also exacerbated hypertension in the Dahl salt-sensitive rat model, which was reversed by oestradiol supplementation⁽¹⁵⁾. The reasons for the apparent discrepancies between the above studies and the present study are not entirely clear. Both the spontaneously hypertensive rat and the Dahl salt-sensitive rat are models of genetic hypertension, and thus may involve different mechanisms than fetal programming models. Presumably, severe protein restriction, as in the present model, could have severely had an impact on developing organs like the cardiovascular system such that it became less responsive to oestradiol.

The present results indicate that the expression of ER α (but not ER β) receptors is reduced in the vasculature of protein-restricted offspring. ER α is shown to play an important role in conveying both vasodilatory^(33,34) and long-term anti-inflammatory actions of oestradiol⁽³⁵⁾. Consistent with this finding of reduced ER α levels in vascular tissues, previous studies have demonstrated an attenuated vasodilatory effect of 17 β -oestradiol in protein-restricted female offspring⁽³⁶⁾. The decreased ER α expression in protein-restricted offspring might be consequent to the decreased oestradiol levels, as the expression of ER α in the vasculature is highly regulated by oestradiol status. However, the finding that oestradiol supplementation does not reverse the vascular ER α levels in protein-restricted offspring suggests that maternal protein restriction may make an impact on or program for permanent reduction in ER α levels, which might contribute to the lack of the cardiovascular protective effect of oestradiol. The mechanisms that contribute to the stable decrease in ER α receptor expression in the vasculature in protein-restricted offspring long after the adverse exposures are gone are not known. Glucocorticoids have been shown to down-regulate ER α expression⁽³⁷⁾. This class of hormones appears to play a central role in mediating the programming effects of under-nutrition in fetal life. Evidence of increased glucocorticoid action in the brain and liver has been demonstrated in both fetal and neonatal rats exposed to low-protein diets *in utero*⁽³⁸⁾, and increased expression of glucocorticoid receptors persists into adult life, mediating hypersensitivity to corticosteroids^(39,40). Therefore, decrease in ER α expression in the protein-restricted offspring influenced by prenatal nutritional status could, in part, be mediated by glucocorticoids. Loss of ER α receptor expression may also be explained by epigenetic modifications of the gene. Methylation in critical

regulatory regions is believed to mark silenced genes. Offspring of dams exposed to low protein during pregnancy or after uteroplacental insufficiency often display anomalous patterns of DNA methylation in the liver of fetuses^(39,41). In the vasculature of protein-restricted females, one potential candidate gene for altered methylation is ER α , which is known to be differentially methylated in response to maternal care during early life⁽⁴²⁾.

Another possible explanation for increased BP in protein-restricted adult female offspring may, in part, be due to endothelial dysfunction observed in adult female offspring of protein-restricted dams^(8,36). Optimal functioning of the NO system is essential for the cardiovascular protective action of oestradiol⁽⁴³⁾. We and others^(8,36) have demonstrated that the endothelial function, endothelial NO synthase expression and NO release are compromised in adult female offspring of protein-restricted dams. Thus, it is possible that the compromised endothelial function, together with reduced ER α levels in protein-restricted female offspring, might have negated the protective effect of oestradiol on BP. This is consistent with human data, which indicate a lack of protective oestrogen effect in hormonal replacement therapy in post-menopausal women who have reduced expression of ER and compromised endothelial function^(44,45).

Although lower oestradiol levels and reduced vascular ER α could have played a permissive role, the possibility of other direct mechanisms that contribute to the development of hypertension cannot be discounted. Studies have shown that testosterone in females may raise BP. The important factor is that the ratio of testosterone to oestradiol is shown to influence BP. In earlier studies^(46,47), elevated testosterone levels in ovariectomised spontaneously hypertensive rats have been shown to cause hypertension. In the present study, protein-restricted adult female offspring have higher testosterone levels and greater testosterone-to-oestradiol ratio (3.5- to 4-fold) compared to controls. These findings and our recent report that hypertension could be abolished by androgen receptor blockade⁽⁶⁾ indicate that testosterone may play a central role in causing hypertension in the females in the present model of prenatal programming by maternal diet. The reason for increased testosterone production in the protein-restricted adult female offspring is not clear; however, similar increases in testosterone levels are reported in the female offspring of pregnant rats exposed to cytokines (IL-6, TNF- α and IL-1 β)^(48,49). Although studies suggest ovaries as a possible source for increased testosterone levels^(48,49), the observation in this study that testosterone levels are also higher in protein-restricted ovariectomised females suggests an extra-ovarian source, possibly adrenal or other peripheral tissues⁽⁵⁰⁻⁵²⁾. There is growing evidence that testosterone stimulates various key components of the systemic and renal renin-angiotensin and endothelin systems while promoting oxidative stress^(53,54). This may exacerbate the progression of renal disease and cardiovascular responses in females⁽⁵⁵⁾. Although the presence of excess testosterone during adulthood may facilitate hypertension to develop in this model, we cannot rule out testosterone playing a programming role during the neonatal period. In this regard, it is important to mention that

the low- birth- weight offspring born to protein-restricted mothers have increased anogenital distances compared to controls^(27,56), suggesting that the fetuses are subjected to androgenic influences during intrauterine life. Further studies are needed to examine the mechanism by which testosterone contributes to the developmental programming of hypertension in females.

It is essential to emphasise the following cautionary remarks regarding the aforesaid interpretations. Although it appears that defects in the sex hormone axis contribute to the observed elevation in arterial pressure, the alterations in sex steroid levels may also be secondary to arterial pressure alterations. Further analysis of longitudinal changes in sex steroid levels and their correlation with BP is essential to establish a cause–effect relationship. In conclusion, these observations suggest that oestradiol plays a major role in limiting the severity of hypertension and in maintaining the normal differences in BP between male and female rats that are developmentally programmed. Failure of oestradiol supplementation to reduce BP in these animals may be due to permanent reduction in the availability of ER and/or increased testosterone levels. The fact that testosterone levels are higher in protein-restricted adult females, together with our previous report of hypertension being abolished with androgen receptor blockade, suggests that testosterone may play an important role in maintaining the elevated BP in this model of maternal protein restriction.

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