Effects of prenatal glucocorticoid exposure on cardiac calreticulin and calsequestrin protein expression during early development and in adulthood

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Overexpression of the conserved Ca^{2+}-binding proteins calreticulin and calsequestrin impairs cardiac function, leading to premature death. Calreticulin is vital for embryonic development, but also impairs glucocorticoid action. Glucocorticoid overexposure during late fetal life causes intra-uterine growth retardation and programmed hypertension in adulthood. To determine whether intra-uterine growth retardation or programmed hypertension was associated with altered calreticulin or calsequestrin expression, effects of prenatal glucocorticoid overexposure (maternal dexamethasone treatment on days 15–21 of pregnancy) were examined during fetal life and postnatal development until adulthood (24 weeks). Dexamethasone (100 or 200 μg/kg of maternal body weight) was administered via osmotic pump. Calreticulin was detected as a 55 kDa band and calsequestrin as 55 and 63 kDa bands in 21 day fetal hearts. Only the 55 kDa calsequestrin band was detected postnatally. Prenatal glucocorticoid overexposure at the higher dose decreased calreticulin protein expression (26 %; $P < 0.05$) but increased calsequestrin protein expression, both 55 and 63 kDa bands, by 87 % ($P < 0.01$) and 78 % ($P < 0.01$); only the 55 kDa calsequestrin band was increased at the lower dose (66 %; $P < 0.05$). Offspring of dams treated at the lower dexamethasone dose were studied further. In control offspring, cardiac calreticulin protein expression declined between 2 and 3 weeks of age, and remained suppressed until adulthood. Cardiac calsequestrin protein expression increased 2-fold between fetal day 21 and postnatal day 1 and continued to increase until adulthood, at which time it was 3.4-fold higher ($P < 0.001$). Prenatal dexamethasone exposure minimally affected postnatal calsequestrin protein expression, but the postnatal decline in calreticulin protein expression was abrogated and calreticulin protein expression in adulthood was 2.2-fold increased ($P < 0.001$) compared with adult controls. In view of the known associations between cardiac calreticulin overexpression and impaired cardiac function, targeted up-regulation of calreticulin may contribute to the increased risk of adult heart disease introduced as a result of prenatal overexposure to glucocorticoids.

Key words: calcium-binding protein, dexamethasone, heart.

INTRODUCTION

Calreticulin is a highly conserved Ca^{2+}-binding protein present in the lumen of the endoplasmic reticulum (ER) of most cell types (reviewed in [1]). Although many unrelated functions have been attributed to calreticulin, its roles in the regulation of Ca^{2+} homoeostasis and as a molecular chaperone of nascent glycoproteins are the two most characterized [1]. Calreticulin's high binding capacity for Ca^{2+} suggests that it may act as a Ca^{2+} store or buffer, and it may represent the main source of inositol 1,4,5-triphosphate (IP_3)-releasable Ca^{2+} [1–4]. Calreticulin may also regulate Ca^{2+} levels directly via interaction with the ER uptake mechanism. Luminal Ca^{2+} released into the cytosol through the IP_3 receptor channel is taken back into the ER by the sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPases (SERCAs). One isoform, SERCA2b, is inhibited by calreticulin, altering the temporal and spatial patterns of IP_3-mediated Ca^{2+} release.

Calreticulin is also vital for the embryonic development of the heart. Calreticulin gene knockout is embryonic-lethal due to impaired cardiac development [5,6]. Comparison of calreticulin-deficient and normal mice has suggested that calreticulin acts as a component of the Ca^{2+}/calcineurin/nuclear factor of activated T-cells (NFAT) transcription pathway during cardiac development [5]. By influencing Ca^{2+} release from the ER, calreticulin regulates the activity of the Ca^{2+}-dependent protein phosphatase calcineurin which, when activated, binds and dephosphorylates members of the NFAT family of transcription factors, resulting in their nuclear translocation [5]. After birth, cardiac calreticulin expression is down-regulated [5]. Since overexpression of calreticulin in the newborn heart (preventing the postnatal decline of calreticulin expression) leads to premature death, such down-regulation appears to be critical for the postnatal maturation of the heart [7].

Calsequestrin is an ER luminal Ca^{2+} binding/storage protein that, like calreticulin, is expressed primarily in cardiac and skeletal muscle [8]. Calreticulin and calsequestrin are structurally similar, even though they exhibit less than 10 % amino acid identity [9–11]. Cardiac-targeted calsequestrin overexpression is also damaging to cardiac function, resulting in left ventricular hypertrophy, which rapidly deteriorates to severe (approx. 2-fold) cardiac hypertrophy and marked left ventricular dysfunction [12]. In skeletal muscle, the major Ca^{2+} storing protein in the ER switches from calreticulin to calsequestrin during development [13]. In rat heart, calsequestrin gene expression has been reported to peak at 4 days after birth [14], although calsequestrin protein expression as determined by immunofluoresence microscopy [15], but not by immunoblot analysis [14], appears to increase with developmental age. Although mice overexpressing calsequestrin survive into adulthood, premature death occurs at 14–16 weeks of age [12]. These effects are observed in conjunction with 1.5-fold up-regulation of calreticulin protein expression [12], together with enhanced NFAT

Abbreviations used: ER, endoplasmic reticulum; GR, glucocorticoid receptor; IP_3, inositol 1,4,5-triphosphate; NFAT, nuclear factor of activated T-cells.

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DNA-binding activities [16]. In addition, calsequestrin overexpression leads to induction of a fetal gene-expression programme, including that of atrial natriuretic factor ('ANF') [12], the secretion of which from the ventricle increases in proportion to the severity of ventricular dysfunction [17].

Epidemiological studies indicate a close association between indices of poor early growth (e.g. birthweight and weight at year 1) and an increased risk of developing insulin resistance, cardiovascular disease and hypertension in adulthood (see [18,19] for recent reviews). Glucocorticoid overexposure during late fetal life is also linked with a low birth weight and programming of hypertension [20,21], raising the possibility that glucocorticoids may interact with calreticulin to programme altered cardiac function. Several studies have indicated that calreticulin impairs glucocorticoid action through interaction with the glucocorticoid receptor (GR). The N-terminus of calreticulin interacts with the DNA-binding domain of GR and prevents it from binding to its specific glucocorticoid-response element [22]. Overexpression of calreticulin in mouse L fibroblasts inhibits glucocorticoid-response-element-mediated transcriptional activation of both a glucocorticoid-sensitive reporter gene and an endogenous, glucocorticoid-sensitive, gene encoding cytochrome P450 [22]. Subsequent studies indicated that the ER form of calreticulin is responsible for inhibition of GR-mediated gene expression, possibly in conjunction with Ca^{2+}/calreticulin-dependent signalling from the ER [23]. These effects are specific to calreticulin, since overexpression of other ER luminal proteins, including calsequestrin, immunoglobulin heavy-chain binding protein (BiP) and ERp72, has no effect on glucocorticoid-sensitive gene expression [23]. Calreticulin has also been implicated in facilitating nuclear export of the GR in mammalian cells via direct contact with the DNA-binding domain of GR [24]. Dexamethasone treatment of LM(TK-) cells doubled the amount of nuclear green fluorescent protein–calreticulin, but did not affect the localization of a green fluorescent protein–calreticulin fusion protein in which the GR-binding N-domain of calreticulin had been deleted. In the present study, we therefore investigated the impact of prenatal exposure to excess glucocorticoids through maternal dexamethasone treatment during the last third of pregnancy to examine potential mechanisms by which adverse effects on cardiac function may arise, focusing on potential modulation of cardiac calreticulin or calsequestrin protein expression.

MATERIALS AND METHODS

Materials

General laboratory reagents were purchased from Sigma (Poole, Dorset, U.K.) with the following exceptions. ECL® reagents, Hyperfilm and secondary antibodies were purchased from Amersham Biosciences (Little Chalfont, Bucks, U.K.). Dexamethasone sodium phosphate was obtained from David Bull Laboratories (Warwick, U.K.). Mini-osmotic pumps were purchased from Charles River (Margate, Kent, U.K.). Anti-calreticulin and anti-calsequestrin rabbit polyclonal IgG were purchased from Upstate Biotechnology (Milton Keynes, U.K.). Cardiac calreticulin knockout *crt*(−/−) and wild-type *crt*(+/+) samples obtained from 18-day-old mouse embryos used to test the specificity of the calreticulin antibody were a kind gift from Professor Marek Michalak (University of Alberta, Edmonton, Alberta, Canada).

Animals

All studies were approved by local ethical review and were conducted in adherence to the regulations of and licensed under the United Kingdom Animal Scientific Procedures Act (1986). Female albino Wistar rats (180–200 g) were purchased from Charles River. Rats were maintained at a temperature of 22 ± 2 °C and subjected to a 12 h:12 h light/dark cycle. Rats were fed on standard, pelleted rodent diet purchased from Special Diets Services (Witham, Essex, U.K.). This diet consisted of 52% carbohydrate, 15% protein, 3% lipid and 30% non-digestible residue by weight, and contained 2.61 kcal/g metabolizable energy. In all experiments, rats were allowed access *ad libitum* to standard diet and water.

Dexamethasone treatment

The fetus is normally protected from higher maternal levels of glucocorticoids by feto-placental 11-β-hydroxysteroid dehydrogenase type-2 ('11 β-HSD2'), which inactivates glucocorticoids [25]. The synthetic glucocorticoid dexamethasone is a poor substrate for this enzyme, and its administration to rats during the last third of pregnancy leads to intra-uterine growth retardation and offspring exhibiting adult hypertension [21,26,27]. In the present experiments, dexamethasone was administered to pregnant rats from day 15 to 21 of gestation by subcutaneous infusion via a chronically implanted osmotic minipump (Alzet Pump Model 2001) at a dose of 100 or 200 μg/kg of maternal body weight per day. The former dose has been used previously to elicit hypertension in the adult offspring [26]. An initial priming dose (0.1 mg) of dexamethasone was also given by subcutaneous injection before minipump implantation. For studies of the persistent effects of maternal dexamethasone treatment on cardiac parameters during postnatal life, dexamethasone was administered at the lower dose only (100 μg/kg of body weight per day; on days 15–21 of gestation). This dose was shown in pilot studies to elicit significant fetal growth retardation in the absence of adverse effects on fetal number or viability. The higher dexamethasone dose was not used, as neonatal viability was decreased. Sham operations involving incision and manipulation under anaesthesia identical to the procedure for implantation of the osmotic minipump were undertaken on control pregnant rats. Control and experimental offspring were weaned at 23 days. Male offspring from at least six separate control and experimental litters were selected for study.

Tissue and blood sampling

Rats were anaesthetized by intraperitoneal injection of sodium pentobarbital (60 mg/ml in 0.9% NaCl; 1 ml/kg of body weight). Once locomotor activity had ceased, hearts were rapidly excised and freeze-clamped using aluminium clamps, which had been pre-cooled in liquid nitrogen. Neonatal rats were killed by decapitation following anaesthesia with sodium pentobarbital. Frozen hearts were stored in liquid nitrogen. Blood was sampled from the chest cavity after the removal of the heart. Blood samples were centrifuged for 5 min at 12000 ***g*** and plasma was stored at −20 °C.

Tissue extraction

Freeze-clamped hearts (approx. 100 mg) were homogenized using a Polytron tissue homogenizer (PT 10 probe; position 5, 15 s) in 1 ml of ice-cold extraction buffer (20 mM Tris, 137 mM NaCl, 2.7 mM KCl, 1 mM $CaCl_2$, 10% glycerol, 1% Igepal, 45 mM Na_3VO_4, 0.2 mM PMSF, 10 μg/ml leupeptin, 1.5 mg/ml benzamidine, 50 μg/ml aprotinin and 50 μg/ml pepstatin A, in DMSO, pH 8.0). Homogenates were then placed on ice for 20 min, centrifuged in an Eppendorf centrifuge (12000 ***g*** for 20 min at

4 °C) and the resulting supernatant was stored at −20 °C until analysis.

Immunoblotting

Heart extracts (25 μg of total protein) were subjected to SDS/PAGE using a 9% resolving gel, with a 6% stacking gel as described previously in [28,29]. Following SDS/PAGE, resolved proteins were transferred electrophoretically to nitrocellulose membranes, and then blocked for 2 h at room temperature in Tris-buffered saline supplemented with 0.05% Tween and 5% (w/v) non-fat powdered milk. The nitrocellulose blots were incubated overnight at 4 °C with polyclonal antisera raised against calreticulin or calsequestrin. They were then washed with Tris-buffered saline supplemented with 0.05% Tween (3 × 5 min) and incubated with horseradish peroxidase-linked secondary anti-rabbit IgG antibody [1:2000; in 1% (w/v) non-fat milk in Tris-buffered saline supplemented with 0.05% Tween] for 2 h at room temperature. Bound antibody was visualized using ECL® according to the manufacturer's instructions. The blots were exposed to Hyperfilm, the signals quantified by scanning densitometry and analysed with Molecular Analyst software (Bio-Rad). The amounts of extracts loaded on to gels were varied to establish that the relative densities of the bands corresponding to calreticulin or calsequestrin were linear with concentration. Immunoblots were performed under conditions in which autoradiographic detection was in the linear response range. Equivalence of protein loading was confirmed by Ponceau S staining. For each panel in each figure, the results are from a single gel exposed for a uniform duration.

Protein determination and statistical analysis

Protein concentrations were determined using the Bradford method with BSA as the standard. The assay was linear over the range of protein concentrations routinely used.

Results are presented as means ± S.E.M., with the numbers of observations in parentheses. Statistical analysis was performed by ANOVA followed by Fisher's *post-hoc* tests for individual comparisons or Student's *t* test as appropriate (Statview; Abacus Concepts, Berkeley, CA, U.S.A.). A P value of < 0.05 was considered to be statistically significant.

RESULTS

Prenatal exposure to dexamethasone impairs intra-uterine growth

Maternal dexamethasone administration from day 15 of pregnancy at doses of 100 and 200 μg/kg of body weight per day led respectively to 20% ($P < 0.01$) and 31% ($P < 0.01$) decreases in fetal body weight at day 21 of gestation (Table 1). Fetal heart weights at day 21 of gestation were also significantly decreased (by 24 and 18% respectively; Table 1). Because of relative protection of heart mass, there was a trend towards increased fetal heart/body weight ratio as a consequence of maternal dexamethasone treatment at the higher dose of 200 μg/kg (Table 1). Maternal dexamethasone administration at the lower dose of 100 μg/kg of body weight per day did not alter gestation length, offspring number or offspring viability (results not shown). In contrast, dexamethasone administration at the higher dose impaired offspring viability. Studies of cardiac calreticulin and calsequestrin protein expression during postnatal life were therefore limited to offspring of dams administered dexamethasone at the lower dose (100 μg/kg of body weight per day).

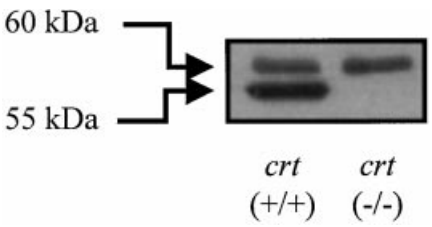

Figure 1 Western blot analysis of homogenates of hearts obtained from 18-day-old embryos from wild-type [*crt*(+/+)] mice and from mice homozygous for calreticulin gene disruption [*crt*(−/−)]

Further details are provided in the Materials and methods section.

Effects of maternal dexamethasone treatment during late pregnancy on cardiac calreticulin and calsequestrin protein expression in the 21-day-old rat fetus

Calreticulin protein expression in fetal hearts was analysed using a rabbit anti-calreticulin antibody from Upstate Biotechnology. To confirm antibody specificity, we performed immunoblot analyses using homogenates of hearts from 18-day-old mouse fetuses homozygous for calreticulin gene disruption [*crt*(−/−)], and compared these with those obtained using homogenates of hearts from 18-day-old wild-type [*crt*(+/+)] mouse fetuses. The calreticulin antibody detected two bands at 55 and 60 kDa in homogenates of hearts from wild-type controls (Figure 1). In contrast, the 55 kDa band was not detected in homogenates of hearts from 18-day-old calreticulin-knockout mouse fetuses, whereas the 60 kDa band continued to be detected (Figure 1). This observation indicates that the 60 kDa band represents binding to an unrelated protein and, therefore, data for the 55 kDa band only are reported. Maternal dexamethasone treat-

Table 1 Body and heart weights of 21-day-old fetuses of control pregnant rats or pregnant rats treated with dexamethasone at a dose of 100 or 200 μg/kg of maternal body weight per day from day 15 of pregnancy

Data are means ± S.E.M. of mean body and heart weights of fetuses obtained from four to five litters in each group (indicated in parentheses). Statistically significant effects of dexamethasone treatment are indicated by $^{*}P < 0.05$ and $^{**}P < 0.01$.

		Dexamethasone-treated	
Maternal group ...	Control	100 μg/kg per day	200 μg/kg per day
Fetal body weight (g)	4.9 ± 0.2 (5)	3.9 ± 0.3** (5)	3.4 ± 0.2** (4)
Fetal heart weight (mg)	34 ± 2 (5)	26 ± 2* (5)	28 ± 2 (4)
Fetal heart weight/fetal body weight (%)	0.69 ± 0.02 (5)	0.67 ± 0.06 (5)	0.83 ± 0.1 (4)

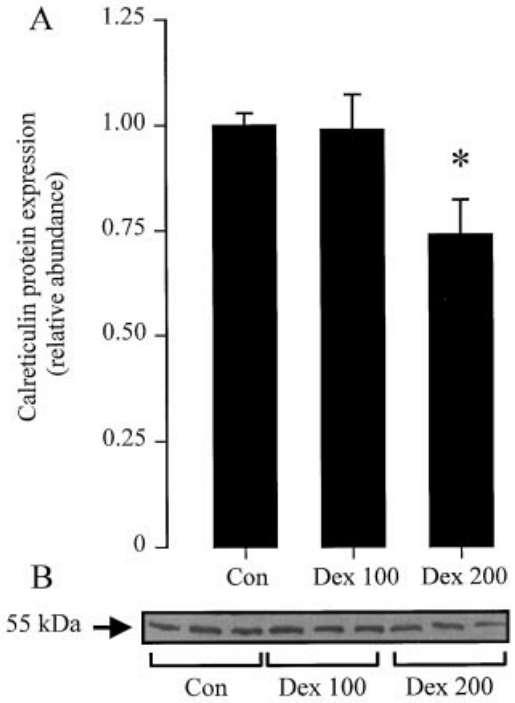

Figure 2 Prenatal exposure to dexamethasone treatment modifies cardiac calreticulin protein expression during late fetal life

Calreticulin protein expression was measured in homogenates of hearts of 21-day-old control fetuses and fetuses from pregnant rats treated with dexamethasone at a dose of 100 (Dex 100) or 200 (Dex 200) μg/kg of maternal body weight per day from day 15 of pregnancy. Quantification of Western analysis of calreticulin protein expression is shown in (**A**) and representative immunoblots are shown in (**B**). Further details are provided in the Materials and methods section. Data are means $\pm$ S.E.M. from six preparations of pooled fetal hearts from separate litters in each group. Statistically significant effects of maternal dexamethasone treatment are indicated by $^{*}P < 0.05$.

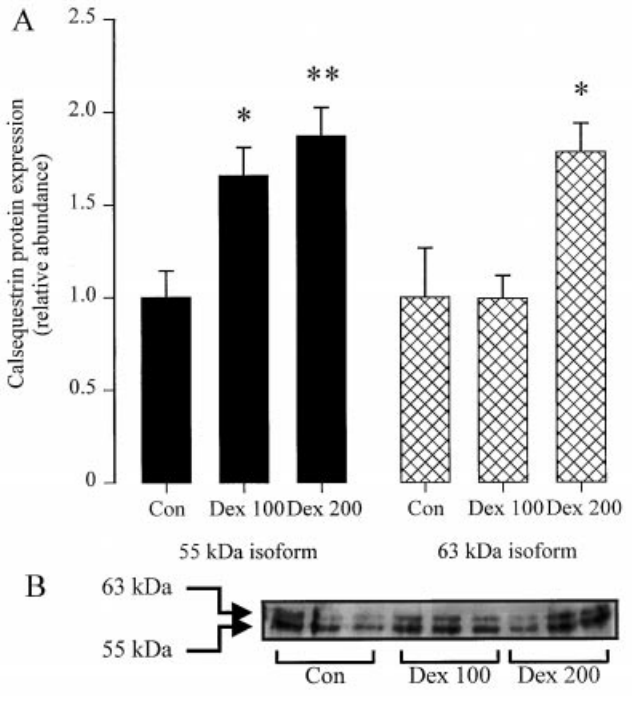

Figure 3 Prenatal exposure to dexamethasone treatment modifies cardiac calsequestrin protein expression during late fetal life

Calsequestrin protein expression was measured in hearts of 21-day-old control fetuses and fetuses from pregnant rats treated with dexamethasone at either 100 (Dex 100) or 200 (Dex 200) μg/kg of maternal body weight per day from day 15 of pregnancy. Quantification of Western analysis of the protein expression of the 55 kDa (closed bars) and 63 kDa (cross-hatched bars) isoforms of calsequestrin is shown in (**A**) and representative immunoblots are shown in (**B**). Further details are provided in the Materials and methods section and the legend to Figure 2. Data are means $\pm$ S.E.M. from six preparations of pooled fetal hearts from separate litters in each group. Statistically significant effects of maternal dexamethasone treatment are indicated by $^{*}P < 0.05$ and $^{**}P < 0.01$.

ment at the higher dose (200 μg/kg of body weight per day) from day 15 to day 21 of gestation significantly decreased calreticulin protein expression (by 26%; $P < 0.05$) in hearts of 21-day-old fetuses (Figure 2). In contrast, maternal dexamethasone treatment at the lower dose (100 μg/kg of body weight per day) from day 15 to day 21 of gestation did not significantly affect calreticulin protein expression in hearts of 21-day-old fetuses (Figure 2).

Calsequestrin exists as a 55 kDa isoform that is present in the adult heart [30] and two 63 kDa (fast and slow) isoforms that are present in adult skeletal muscle [31]. In the present study, the calsequestrin antibody recognized two bands of molecular masses of 55 and 63 kDa in hearts of 21-day-old fetuses (Figure 3). Thus the data demonstrate that both the 55 and 63 kDa isoforms of calsequestrin are present in the hearts of neonatal rats, with approximately equal distribution between the two bands. Protein expression of the 55 kDa calsequestrin isoform in the 21-day-old fetal heart was significantly increased by maternal dexamethasone treatment from day 15 to day 21 of gestation at both the lower (by 66%; $P < 0.05$) and higher (by 87%; $P < 0.01$) dexamethasone doses (Figure 3A). In contrast, protein expression of the 63 kDa calsequestrin isoform in the 21-day-old fetal heart was not affected by maternal dexamethasone treatment at the lower dose, but was significantly increased (78%; $P < 0.01$) by maternal dexamethasone treatment at the higher dose (Figure 3B).

Calreticulin and calsequestrin protein expression exhibit divergent patterns in the heart during postnatal development in control rats

Cardiac calreticulin protein expression was maintained relative to the 21-day-fetal level during the first 2 weeks after birth (Figure 4A). Cardiac calreticulin protein expression subsequently declined significantly ($P < 0.05$) between 2 and 3 weeks of age to 59% of the level of protein expression observed in hearts of 21-day-old fetuses (Figure 4A). Cardiac calreticulin protein expression continued to be suppressed relative to the level of expression observed in hearts of 21-day-old fetuses at 6 (by 43%; $P < 0.05$), 12 (by 54%; $P < 0.01$) and 24 (by 56%; $P < 0.01$) weeks of age (Figure 4A). The postnatal profile for calsequestrin protein expression was quite distinct from that of calreticulin (Figure 4B). The calsequestrin antibody strongly recognized a single band corresponding to a molecular mass of 55 kDa in hearts of postnatal rats, although a 63 kDa band was faintly detectable for up to 2 weeks after birth (Figure 4B). Cardiac calsequestrin protein expression was increased by 2.0-fold relative to the 21-day-fetal level of protein expression at day 1 after birth, and was significantly ($P < 0.05$) higher at 1 (by 2.4-fold), 2 (by 2.5-fold) and 3 (by 2.7-fold) weeks of age relative to the 21-day-fetal level (Figure 4B). Cardiac calsequestrin protein expression in control offspring increased only modestly (by 26%; not significant) between 3 and 24 weeks of age, such that at 24 weeks of age cardiac calsequestrin protein expression was 3.4-fold

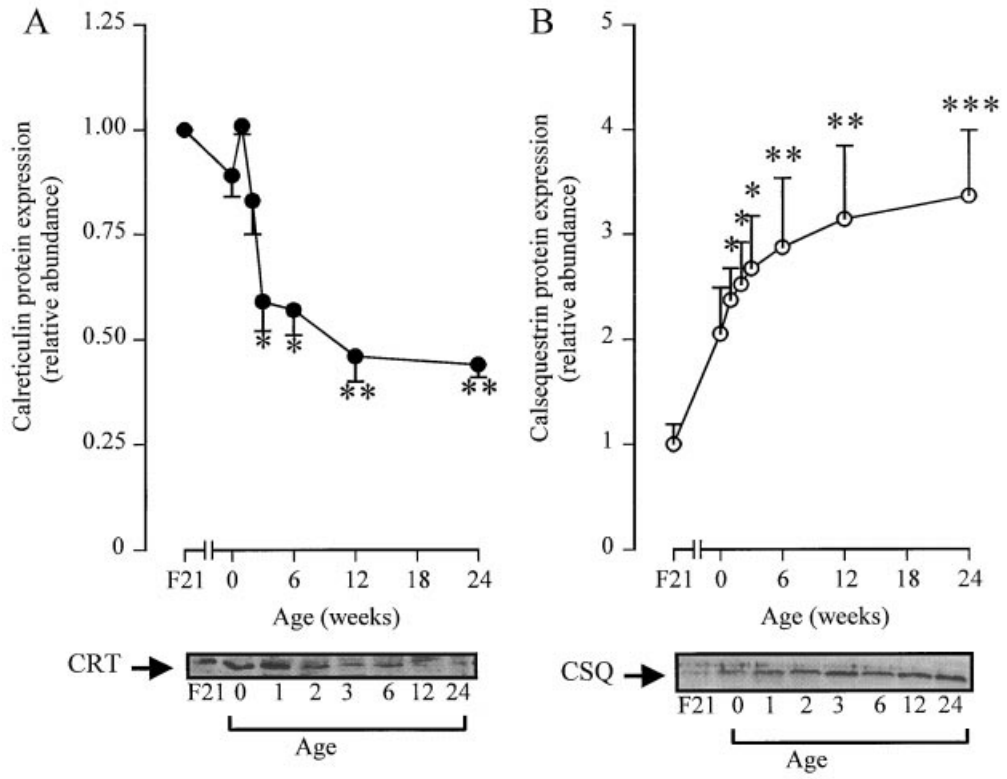

Figure 4 Calreticulin and calsequestrin protein expression exhibit divergent patterns in the heart during postnatal development in control rats

Calreticulin and calsequestrin protein were measured in hearts of control offspring at intervals during postnatal development (0, 1, 3, 6 and 12 weeks of age) until adulthood (24 weeks of age) and expressed relative to protein expression in control 21-day-old fetuses (F21). Quantifications of Western analysis of calreticulin and calsequestrin protein expression are shown in (**A**) and (**B**) respectively. For both calreticulin and calsequestrin, only the lower (55 kDa) band was quantified. Representative immunoblots for calreticulin (CRT) and calsequestrin (CSQ) protein expression are also shown. Further details are provided in the Materials and methods section. Data are means ± S.E.M. from six preparations from individual rats in each group. Statistically significant differences from the level of protein expression in control 21-day-old fetuses are indicated by $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

higher ($P < 0.001$) relative to the 21-day-fetal level (Figure 4B). Thus as observed in skeletal muscle [13], the present data confirm earlier studies [15] demonstrating that the heart is normally characterized by a relative shift in the major Ca^{2+}-storing protein in the ER from calreticulin to calsequestrin during development to adulthood.

Prenatal overexposure to dexamethasone selectively modifies the pattern of cardiac calreticulin protein expression during postnatal development

Analysis of cardiac calreticulin protein expression in offspring exposed to glucocorticoid excess *in utero* revealed that, as in the control offspring, little change in cardiac calreticulin protein expression relative to the 21-day-fetal level in dexamethasone-treated dams was detectable during the first 2 weeks of age (Figure 5). However, the developmental decline in cardiac calreticulin protein expression observed between 2 and 3 weeks of age in control offspring was abrogated in offspring of dams administered dexamethasone (compare Figures 4 and 5). Furthermore, in contrast with the persistent decline in cardiac calreticulin protein expression observed in control offspring up to 24 weeks of age (Figure 4), cardiac calreticulin protein expression in offspring of dams administered dexamethasone significantly increased (by 35 %; $P < 0.05$) between 6 and 12 weeks of age, and continued to increase between 12 and 24 weeks of age (Figure 5). As a result, cardiac calreticulin protein expression was significantly higher at 12 (by 1.6-fold; $P < 0.01$) and 24 weeks of age (by 1.9-fold; $P < 0.001$) relative to the level of protein expression in 21-day-old fetuses of dexamethasone-treated dams (Figure 5A). We subsequently analysed the extent to which exposure to excess glucocorticoids *in utero* influenced cardiac calreticulin protein expression by directly comparing hearts of control 24-week-old offspring and 24-week-old offspring of dams administered dexamethasone on days 15–21 of gestation on the same immunoblot (Figure 6). Cardiac calreticulin protein expression in offspring of dexamethasone-treated dams was significantly higher (by 2.2-fold; $P < 0.001$) compared with control offspring at 24 weeks of age.

Analysis of cardiac calsequestrin protein expression in offspring exposed to glucocorticoid excess *in utero* revealed that cardiac calsequestrin protein expression, relative to the 21-day-fetal level, did not increase as dramatically as in control offspring (Figure 5B), possibly reflecting the fact that cardiac calsequestrin protein expression was already elevated by exposure to excess glucocorticoids at fetal day 21 (Figure 3). Nevertheless, cardiac calsequestrin protein expression in offspring of dams administered dexamethasone tended to increase relative to the 21-day-fetal level at 1, 3, 6 and 12 weeks of age (by 40–57 %; not significant), and was significantly increased (by 89 %; $P < 0.05$) at 24 weeks of age (Figure 5B). Direct comparison of calsequestrin protein expression in hearts of control 24-week-old offspring and 24-week-old offspring of dams administered dexamethasone from day 15 to day 21 of gestation on the same immunoblot

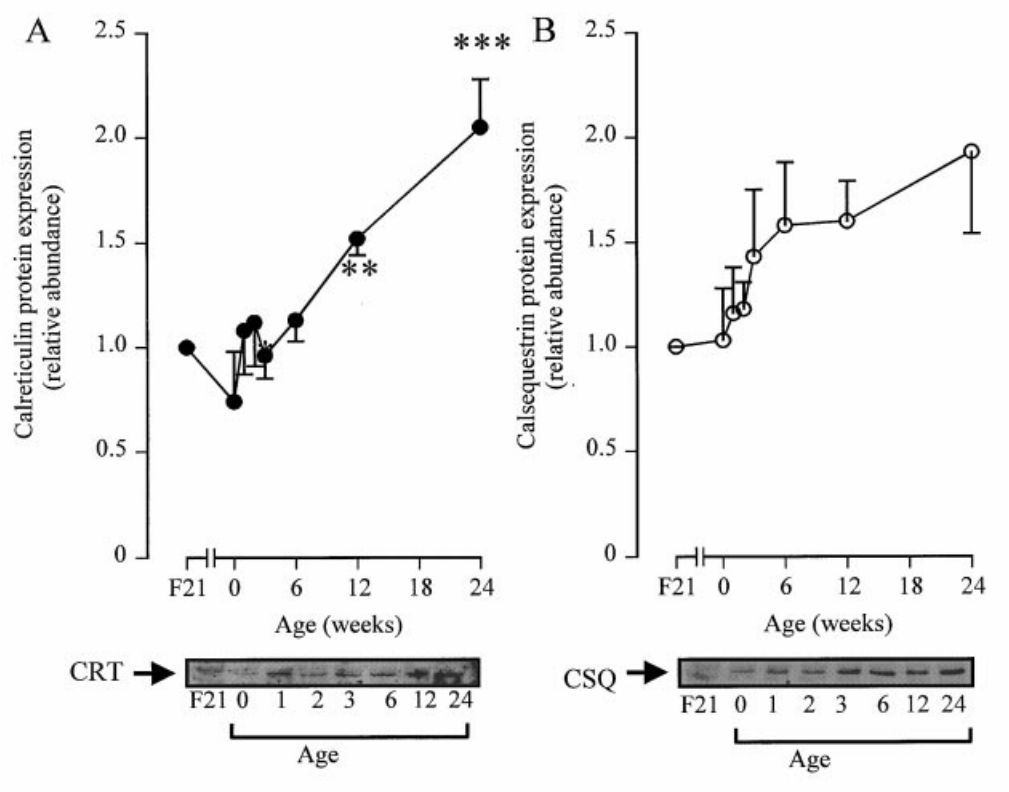

Figure 5 Prenatal overexposure to dexamethasone selectively modifies the pattern of cardiac calreticulin protein expression during postnatal development

Cardiac calreticulin and calsequestrin protein were measured in offspring of dexamethasone-treated pregnant rats at intervals during postnatal development (0, 1, 3, 6 and 12 weeks of age) until adulthood (24 weeks of age) and expressed relative to protein expression in 21-day-old fetuses of dexamethasone-treated dams (F21). Quantifications of Western analysis of calreticulin and calsequestrin protein expression are shown in (**A**) and (**B**) respectively, together with representative immunoblots. Further details are provided in the Materials and methods section and the legend to Figure 4. Data are means $\pm$ S.E.M. from six preparations from individual rats in each group. Statistically significant differences from the level of protein expression in 21-day-old fetuses of dexamethasone-treated dams are indicated by $^{**}P < 0.01$ and $^{***}P < 0.001$.

revealed that, although the postnatal increase in cardiac calsequestrin protein expression relative to fetal day 21 was lower in offspring of dexamethasone-treated dams compared with control offspring, no statistically significant differences in cardiac calsequestrin protein expression existed between control offspring and offspring of dexamethasone-treated dams at 24 weeks of age. Previous studies have also demonstrated that calsequestrin protein expression (expressed either relative to total protein or normalized to β-tubulin protein expression) is not altered in hypertrophied or failing human hearts compared with non-failing human heart tissue [32]. Consequently, it can be concluded that overexposure to glucocorticoids *in utero* specifically affected the postnatal response of calreticulin protein expression by abrogating the normal decline in expression observed in the immediate postnatal period and actually increasing cardiac calreticulin protein expression in adulthood.

DISCUSSION

The present study identified significant calreticulin and calsequestrin protein expression in the fetal (day 21) rat heart. Reciprocal changes in protein expression in response to glucocorticoid overexposure are identified during late fetal development. Detailed time courses of changes in cardiac calreticulin and calsequestrin protein expression during postnatal development suggest that the period between 2 and 3 weeks of age in the rat is critical for suppressing cardiac calreticulin expression. We show that the developmental rise in calsequestrin expression during the first week of postnatal life in the rat precedes the developmental decline in calreticulin protein expression, suggesting independent regulation of calreticulin and calsequestrin protein expression postnatally. Importantly, we demonstrate that the normal developmental decline in cardiac calreticulin protein expression is abrogated, and cardiac calreticulin protein expression in adulthood significantly enhanced, as a consequence of prenatal dexamethasone exposure. The postnatal expression of calsequestrin protein in the heart is little affected by prenatal dexamethasone exposure. Given the adverse impact of calreticulin overexpression on cardiac function demonstrated by others, we propose that targeted up-regulation of cardiac calreticulin expression is a potential candidate mechanism responsible for the increased risk of adult heart disease introduced as a result of prenatal overexposure to glucocorticoids.

The calreticulin antibody used detected two bands at 55 and 60 kDa in homogenates of hearts from wild-type controls. The 60 kDa band, but not the 55 kDa band, was also detected in homogenates of hearts from 18-day-old calreticulin-knockout mouse fetuses, suggesting that the 60 kDa band represents non-specific binding. Cardiac calreticulin protein expression was maintained relative to the 21-day-fetal level during the first 2 weeks after birth, but subsequently declined between 2 and 3 weeks of age, and continued to be suppressed into adulthood.

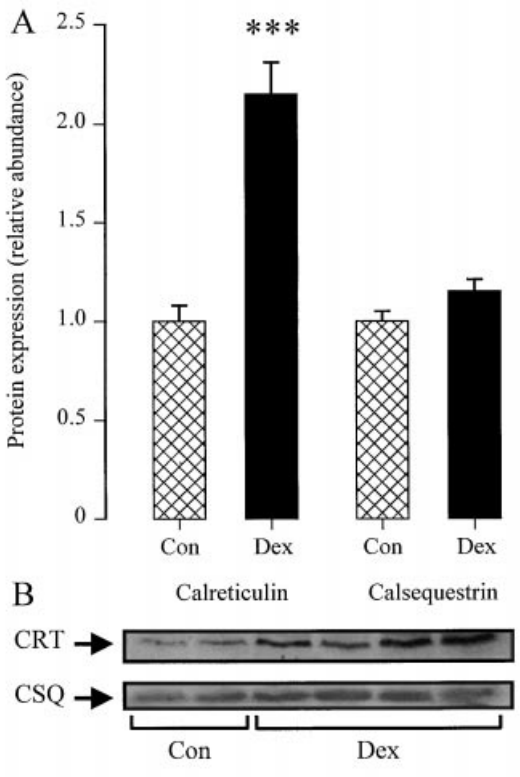

Figure 6 Prenatal overexposure to dexamethasone selectively alters cardiac calreticulin protein expression in 24-week-old offspring

Calreticulin and calsequestrin protein were measured in homogenates of hearts of 24-week-old control offspring (Con) and offspring of dexamethasone-treated pregnant rats (Dex) and expressed relative to protein expression in control offspring. Quantification of Western analysis of calreticulin and calsequestrin protein expression is shown in (**A**) and representative immunoblots are shown in (**B**). Further details are provided in the Materials and methods section and the legend to Figure 4. Data are means ± S.E.M. from six preparations from individual rats in each group. Statistically significant effects of prenatal overexposure to dexamethasone are indicated by $***P < 0.001$.

The present data extend earlier studies demonstrating high calreticulin expression in the heart during early embryonic development [5,15], but down-regulation in late embryonic stages and maintenance at very low levels in the mature heart [5,15,33–35].

A previous study in the rat demonstrated a marked decline in cardiac calreticulin protein expression at birth compared with fetal day 14 [15], implying a progressive decline in cardiac calreticulin protein expression during the last third of pregnancy. We identified a dose-dependent decrease in calreticulin protein expression in the 21-day-old fetal heart associated with prenatal dexamethasone exposure. Other studies have indicated that calreticulin impairs glucocorticoid action through interaction with the GR. The ER form of calreticulin inhibits GR-mediated gene expression, possibly in conjunction with Ca^{2+}/calreticulin-dependent signalling from the ER [23]. In addition, calreticulin may promote nuclear export of the GR [24]. Consequently, dexamethasone-induced down-regulation of cardiac calreticulin in fetal hearts in late gestation may enhance glucocorticoid action and/or impair nuclear export of the GR.

Transgenic mice overexpressing calreticulin do not exhibit any obvious gross abnormalities during the first 1 or 2 weeks of life. This may reflect the observation in the present study that cardiac calreticulin protein expression remains high during this period. However, decreased systolic function and sudden cardiac death occur from 15 days after birth [7]. This period coincides with the precipitous decline in cardiac calreticulin protein expression during the third week of life observed in the present study. Taken together, the findings suggest that that the period between two and three weeks of age in the rat is critical for suppressing cardiac calreticulin expression and, therefore, function. Importantly, we show that the normal developmental decline in cardiac calreticulin protein expression between 2 and 3 weeks of age in the rat is abrogated as a consequence of prenatal dexamethasone exposure. The degree of calreticulin overexpression (< 2-fold) observed in the offspring of dexamethasone-treated dams in the present study is much lower than that observed in transgenic mice overexpressing calreticulin at 2–3 weeks of age (approx. 15–20-fold) [7]. This may explain why this effect does not lead to premature death over this period. The degree of cardiac calreticulin protein expression continued to modestly increase with age in rats subjected to prenatal dexamethasone treatment and was 2.2-fold higher than normal in adulthood at 24 weeks of age.

While not lethal, this degree of calreticulin overexpression may have functional consequences. Functional consequences of cardiac calreticulin overexpression in the calreticulin transgenic animals, which exhibit 20-fold increases in the level of calreticulin at 14 days of age, include decreased systolic function, sinus bradycardia and prolonged atrioventricular node conduction [7]. A modest (1.6-fold) overexpression of calreticulin in a mouse L fibroblast cell line, comparable with that demonstrated in the present study in the heart, increases intracellular Ca^{2+} storage and decreases store-operated Ca^{2+} influx [4]. Our results thus raise the possibility of a direct alteration in cardiac Ca^{2+} homoeostasis as a consequence of targeted up-regulation of calreticulin. We and others have already established that prenatal dexamethasone treatment leads to the development of hypertension in adulthood [20,21]. Altered cardiac Ca^{2+} homoeostasis secondary to calreticulin overexpression may be an important additional component contributing to the development of cardiovascular disease arising as a consequence of prenatal dexamethasone treatment, and physiological measurements to gain insight into the impact of cardiac calreticulin overexpression induced by prenatal dexamethasone exposure on heart function would be of considerable interest.

The calsequestrin antibody that we used recognized two bands of molecular masses of 55 and 63 kDa in hearts of 21-day-old fetuses. Postnatally, the calsequestrin antibody strongly recognized a single band corresponding to a molecular mass of 55 kDa in hearts of postnatal rats, although a 63 kDa band was faintly detectable for up to 2 weeks after birth. Similar observations have been reported by Imanaka-Yoshida and co-workers [15], who detected two calsequestrin-immunoreactive bands (at 54 and 62 kDa) in 0-day-old neonatal rat hearts, with the 62 kDa protein becoming faint in hearts of 3-week-old rats. Calsequestrin is a key component of the regulation of Ca^{2+} release during cardiac muscle excitation/contraction coupling in the adult heart (reviewed in [36]). We identified a dose-dependent effect of prenatal dexamethasone to up-regulate the protein expression of both calsequestrin isoforms in the 21-day-old fetal heart. It is thus possible that the reciprocity of cardiac calreticulin and calsequestrin protein expression observed in late fetal life in response to prenatal dexamethasone results in a functional shift in the regulation of cardiac ER Ca^{2+} release; however, this remains to be established. Glucocorticoids are known to increase during late pregnancy to modify fetal tissue structure and function in preparation for birth [37]. Since cardiac calreticulin protein expression was suppressed but cardiac calsequestrin

protein expression enhanced by antenatal dexamethasone, it seems likely that the maternal glucocorticoid surge normally observed in late gestation participates in orchestrating changes in relative cardiac expression of calreticulin and calsequestrin in the late fetal heart in preparation for birth. As cardiac-specific overexpression of calsequestrin in the mouse heart leads to cardiac hypertrophy in transgenic mice [16,38], enhanced relative expression of calsequestrin may contribute to the relative protection of fetal heart mass found after high-dose dexamethasone treatment.

The postnatal developmental profile of calsequestrin was compared directly with that of calreticulin. In a previous study [15], no marked changes in cardiac calsequestrin protein expression were detected between 0 day-old neonatal and 3 week-old rat hearts. However, Lompre et al. [14] reported that the calsequestrin mRNA level peaks at 4 days after birth, whereas others [39] reported an increase in calsequestrin mRNA expression during rabbit heart development. We identified a gradual increase in cardiac calsequestrin protein expression during the first 3 weeks of postnatal life. Cardiac calsequestrin protein expression remained relatively unchanged thereafter. The developmental rise in calsequestrin expression during the first week of postnatal life in the rat preceded the developmental decline in calreticulin protein expression from 2 to 3 weeks of postnatal development. It is noteworthy that the effect of prenatal dexamethasone to modify cardiac calreticulin protein expression during early postnatal development was not accompanied by any major modification of cardiac calsequestrin protein expression. Similarly, molecular assessment of cardiomyocyte gene expression in transgenic mice overexpressing calreticulin revealed no change in calsequestrin expression [7]. Thus, our data are consistent with the concept of independent regulation of cardiac calreticulin and calsequestrin protein expression during postnatal life.

A low birth weight has been taken to reflect adverse developmental influences during early life that contribute to an increased risk of developing hypertension and heart disease. Maternal dexamethasone treatment during the last third of pregnancy in the rat causes intra-uterine growth retardation in association with adulthood hypertension in the offspring [21,40, 41]. In view of the known associations between cardiac calreticulin overexpression and impaired cardiac function, targeted up-regulation of calreticulin may contribute to the increased risk of adult heart disease introduced as a result of prenatal overexposure to glucocorticoids.

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