Early growth retardation induced by excessive exposure to glucocorticoids *in utero* selectively increases cardiac GLUT1 protein expression and Akt/protein kinase B activity in adulthood

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Abstract

In the rat, dexamethasone treatment during late pregnancy leads to intrauterine growth retardation and is used as a model of early programming of adult onset disease. The present study investigated whether pre-natal dexamethasone treatment modifies cardiac glucose transporter (GLUT) protein expression in adulthood and identified signalling pathways involved in the response. Dexamethasone (100 µg/kg body wt per day) administered via an osmotic pump to pregnant rats (day 15 to day 21; term = 22 to 23 days) reduced fetal weight at day 21 and caused hypertension, hyperinsulinaemia and elevated corticosterone levels in the adult (24-week-old) male offspring. Cardiac GLUT1 protein expression was selectively up-regulated (2.5-fold; $P < 0.001$), in the absence of altered cardiac GLUT4 protein expression, in adult male offspring of dexamethasone-treated dams. Maternal dexamethasone treatment did not influence cardiac GLUT1 protein expression during fetal or early post-natal life. We examined potential regulatory signalling proteins that might mediate

up-regulation of cardiac GLUT1 protein expression in adulthood. We observed marked (2.2-fold; $P < 0.01$) activation of Akt/protein kinase B (PKB), together with modest activation of the anti-apoptotic protein kinase C (PKC) isoforms PKC α (88%, $P < 0.05$) and PKC ϵ (56%, $P < 0.05$) in hearts of the early-growth-retarded male offspring. These effects were, however, observed in conjunction with up-regulation of cardiac protein expression of PKC β_1 (191%, $P < 0.01$), PKC β_2 (49%, $P < 0.05$) and PKC δ (35%; $P < 0.01$), effects that may have adverse consequences. Maternal dexamethasone treatment was without effect on cardiac extracellular signal-related kinase (ERK) 1 or ERK2 activity in adulthood. In conclusion, our data demonstrate an effect of maternal dexamethasone treatment to up-regulate cardiac GLUT1 protein expression in early-growth-retarded, hypertensive, hyperinsulinaemic adult male offspring, an effect observed in conjunction with activation of Akt/PKB.

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Introduction

Under resting conditions, the adult heart derives about 70% of its energy from the oxidation of lipids, and the remainder primarily from glycolysis and glucose oxidation (Taegtmeyer 1994). However, specific situations exist that are known to be associated with increased cardiac glucose utilisation: these include late fetal life (Santalucia *et al.* 1992), myocardial ischaemia (Brosius *et al.* 1997) and pressure-overload-induced cardiac hypertrophy (Bishop & Altschuld 1970, Taegtmeyer & Overturf 1988, Christie & Rodgers 1994). Glucose transport is rate limiting for cardiac glucose metabolism. Glucose enters the heart via two facilitative glucose transporters GLUT1 and GLUT4 (Mueckler 1990). GLUT1 is a major mediator of basal cardiac glucose uptake (Kraegen *et al.* 1993, Laybutt *et al.* 1997), and normally accounts for approximately 30% of

the total cardiac glucose transporters in the adult heart (Fischer *et al.* 1997). The contribution of GLUT1 to total cardiac glucose transporter number is even higher in late fetal life and early neonatal life, when GLUT4 protein expression is relatively low (Studelska *et al.* 1992, Postic *et al.* 1994). However, the relative contribution of the two glucose transporters changes post-natally when GLUT1 protein expression is down-regulated (Studelska *et al.* 1992, Postic *et al.* 1994), whereas that of GLUT4 is up-regulated (Studelska *et al.* 1992, Postic *et al.* 1994). The post-natal down-regulation of GLUT1 has been attributed in part to reduced protein abundance of the zinc finger transcription factor Sp1 (Santalucia *et al.* 1999).

Impaired growth and development during fetal life and infancy in man has been associated with an increased risk for the later development of hypertension, insulin resistance syndrome (Syndrome X) and adverse coronary events

(reviewed by Barker 1999, 2000). Excessive prenatal exposure to glucocorticoids has been suggested as a possible underlying mechanism (Benediktsson *et al.* 1993). In the rat, the administration of the synthetic glucocorticoid dexamethasone during the last third of pregnancy leads to intrauterine growth retardation in conjunction with hypertension (Levitt *et al.* 1996, reviewed by Barker 1999) and glucose intolerance (Nyirenda *et al.* 1998) in later life, a pattern paralleling that found in man. It has been proposed that prenatal exposure to excessive glucocorticoids up-regulates glucocorticoid activity throughout life by decreasing the expression of hippocampal glucocorticoid receptors, thereby blunting feedback control of glucocorticoid secretion (Levitt *et al.* 1996). Increased myocardial glucose uptake is observed in conjunction with increased cardiac GLUT1 expression in spontaneously hypertensive rats (Paternostro *et al.* 1995). Long-term insulin treatment also up-regulates cardiac GLUT1 protein expression (Laybutt *et al.* 1997), and sustained hyperinsulinaemia is one of the major characteristics of the insulin resistance syndrome (reviewed by Reaven 1995). Thus, at least two of the pathophysiological features associated with impaired early growth (hypertension and hyperinsulinaemia) are associated with up-regulation of cardiac GLUT1 protein expression.

In some, but not all, studies, mitogen-activated protein kinases (MAPKs) have been identified as being involved in the cardiac hypertrophic response: these include p42/p44 extracellular signal-related kinases (ERK1 and ERK2) (Thorburn *et al.* 1994a,b, Post *et al.* 1996, Ramirez *et al.* 1997, Zechner *et al.* 1997, Choukroun *et al.* 1998, Nemoto *et al.* 1998, reviewed by Sugden 1999). Recent evidence has demonstrated that increased MAPK activity elicits greatly increased expression of GLUT1 in adipocytes (Yamamoto *et al.* 2000). Thus, MAPKs may co-ordinate increased cardiac GLUT1 expression with the overall cardiac hypertrophic response. Other studies have demonstrated a possible involvement of members of the protein kinase C (PKC) superfamily, in that chronic pressure overload activates PKC α and PKC ϵ (Paul *et al.* 1997, Takeishi *et al.* 1999): once activated, PKCs are thought to directly enhance hypertrophic gene expression by acting on transcription factors such as serum response factor and Sp 1 (Sadoshima & Izumo 1993b, Karns *et al.* 1995). Finally, recent studies have suggested that the multifunctional protein kinase Akt/protein kinase B (Akt/PKB) can regulate protein synthesis (and therefore cell growth) in response to insulin in cardiomyocytes from adult (Wang *et al.* 2000) and neonatal (Pham *et al.* 2000) rats *in vitro*. In view of the changes in cardiac GLUT1 expression observed during normal cardiac development and response to chronic hyperinsulinaemia and hypertension, we investigated whether prenatal dexamethasone treatment is associated with early or persistent changes in cardiac GLUT1 protein expression. Comparison was made with cardiac GLUT4 protein expression. In addition,

given that common signals may be involved in the regulation of cardiac GLUT1 expression during cardiac development and in response to hyperinsulinaemia or hypertension, we investigated possible intracellular signalling mechanisms whereby altered cardiac GLUT1 expression might be mediated.

Materials and Methods

Materials

All organic solvents were of analytical grade and obtained from BDH Laboratories Supplies (Poole, Dorset, UK). General laboratory reagents were from Sigma Chemical Co. Ltd (Poole, Dorset, UK), with the following exceptions. ECL reagents, Hyperfilm and secondary antibodies were purchased from Amersham International plc (Amersham, Bucks, UK). Anti-GLUT1 and anti-GLUT4 polyclonal immunoglobulin G (IgG) antibodies and anti-PKC $\beta_{1/2}$, anti-PKC δ and anti-PKC α polyclonal IgG antibodies were purchased from Insight Biotechnology Ltd (Wembley, Middlesex, UK). Anti-PKC ϵ monoclonal IgG antibody was purchased from Transduction Labs. (Lexington, KY, USA). Anti-phospho-MAPK and anti-phospho-Akt/PKB were purchased from New England Biolabs Ltd (Hitchin, Herts, UK). Dexamethasone (sodium phosphate) was obtained from David Bull Laboratories (Warwick, UK). Bradford reagents were purchased from BioRad Ltd (Hemel Hempstead, Herts, UK). Kits for the measurement of corticosterone were purchased from ICN Pharmaceuticals, Inc. (Basingstoke, Hants, UK). Kits for determination of plasma insulin concentrations were from Phadesepp Pharmacia (Uppsala, Sweden). Alzet osmotic mini-pumps were purchased from Charles River (Margate, Kent, UK).

Animals

All studies were conducted in adherence to the regulation of the United Kingdom Animal Scientific Procedures Act (1986). Female albino Wistar rats (200–220 g) were purchased from Charles River. Rats were maintained at a temperature of $22 \pm 2^\circ\text{C}$ and subjected to a 12 h light/12 h darkness cycle. Rats were maintained on standard, pelleted rodent diet purchased from Special Diet Services (Witham, Essex, UK). This diet consisted of 52% carbohydrate, 15% protein, 3% lipid and 30% non-digestible residue (by wt), and contained 2.61 Kcal/g metabolisable energy. In all experiments, rats were allowed access *ad libitum* to standard diet and water.

Tissue and blood sampling

All studies were undertaken with rats in the absorptive state (between 1000–1100 h). Rats were anaesthetised by

injection of sodium pentobarbital (60 mg/ml in 0.9% NaCl; 1 ml/kg body wt i.p.) and, once locomotor activity had ceased, hearts were rapidly excised and freeze-clamped using aluminium clamps pre-cooled in liquid nitrogen. Frozen hearts were stored in liquid nitrogen. Blood was sampled from the chest cavity after the removal of the heart. Blood was sampled from fetuses after decapitation and from neonatal rats after decapitation following anaesthesia by injection of sodium pentobarbital (60 mg/ml in 0.9% NaCl; 1 ml/kg body wt, i.p.). Blood samples were centrifuged for 5 min at 12 000 g and plasma was stored at -20°C .

Dexamethasone administration

Dexamethasone was administered to pregnant rats from day 15 of gestation by subcutaneous infusion via a chronically-implanted osmotic minipump, at a dose of 100 µg/kg body wt per day. This dosage has been used previously to elicit hypertension in the adult offspring (Levitt *et al.* 1996); the protocol used in the present experiments differs from that used previously in that in our studies dexamethasone was infused at a steady rate, whereas in the previous study, dexamethasone was administered daily via a single bolus injection. An initial priming dose (0.1 mg) of dexamethasone was also given by subcutaneous injection before minipump implantation. The minipumps were not removed at birth to avoid handling and anaesthesia of the dams. Undue stress immediately post partum can lead to abandonment of the litter, as well as to interrupted suckling over a critical developmental period. The minipumps were set to deliver a fixed volume that was exhausted after 7 days. Sham operations involving incision and manipulation under anaesthesia identical to the procedure for implantation of the osmotic minipump were undertaken on control pregnant rats.

Western blotting

Cardiac samples (approx. 100 mg) were homogenised using a Polytron Tissue homogeniser (PT 10 probe; position 5, 15 s) in 1 ml ice-cold extraction buffer A (20 mM Tris, 137 mM NaCl, 2.7 mM KCl, 1 mM CaCl_2 , 10% glycerol, 1% Igepal, 45 mM sodium orthovanadate, 0.2 mM PMSF, 10 µg/ml leupeptin, 1.5 mg/ml benzamide, 50 µg/ml aprotinin, 50 µg/ml pepstatin A (in DMSO), pH 8.0). Homogenates were placed on ice for 20 min, centrifuged in an Eppendorf centrifuge (12 000 r.p.m. for 20 min at 4°C) and the supernatants stored (-20°C) until analysis. Protein concentrations were determined by the method of Bradford (1976) using bovine serum albumin (BSA) as standard. The assay was linear over the range of protein concentrations routinely used. Samples (20–50 µg total protein) were subjected to SDS-PAGE using either a 9% or a 12% resolving gel

(depending on the molecular weight of the protein to be studied), with a 6% stacking gel. Following SDS-electrophoresis, resolved proteins were transferred electrophoretically to nitrocellulose membranes, and then blocked for 2 h at room temperature with Tris-buffered saline (TBS) 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 each of the glucose transporters. Phospho-PKC α , phospho-PKC ϵ , and phospho-Akt/PKB were used in a 1:100 dilution; anti-GLUT1 and anti-GLUT4 were used in a 1:150 dilution and PKC α , PKC $\beta_{1/2}$, PKC δ , PKC ϵ and PKC ζ in a 1:500 dilution. The blots were washed with TBST (TBS with 0.05% Tween 20) (3×5 min) and incubated with horseradish peroxidase-linked secondary antibody IgG anti-rabbit (1:2000 in 1% (wt/vol.) non-fat milk in TBST) or horseradish peroxidase-linked secondary antibody IgG anti-mouse (1 in 2000) for 2 h at room temperature. Bound antibody was visualised using ECL according to the manufacturer's instructions. The blots were then exposed to Hyperfilm and the signals quantified by scanning densitometry and analysed with Molecular Analyst software (Biorad Ltd). An initial characterisation using competing antigenic peptides was performed to determine the specificity of the antibodies. Some antibodies recognised more than one protein, but in each case specific protein bands were identified by their specific reduction in immunoreactivity when competing peptide was included. The amounts of extracts loaded on to the gel were varied to establish that the relative densities of the bands corresponding to the individual proteins 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. Each representative blot depicts a single gel exposed for a uniform duration.

Blood pressure measurements

Systolic blood pressures were determined by tail cuff plethysmography at room temperature in lightly anaesthetised (sodium pentobarbital; 35 mg/kg body wt) rats. All measurements were undertaken in the morning, and each rat had five blood pressure traces recorded in a single session. The coefficient of variation was 3.2%. Blood pressures were quantified using a photoelectric sensor attached to the blood pressure monitor, in conjunction with software designed to determine pressures.

Hormone and metabolite measurements

Plasma insulin and corticosterone concentrations were measured by radioimmunoassay using commercial kits (Phadeseph Pharmacia and ICN Pharmaceuticals Inc. respectively). In the corticosterone radioimmunoassay, the

antiserum used showed <0.05% cross reactivity with cortisol and <0.01% cross reactivity with dexamethasone. It is appreciated that corticosterone levels in blood sampled from the chest cavity after terminal anaesthesia may not represent true basal corticosterone levels; however, every effort was made to standardise procedures and time taken for sampling between the experimental groups and thus values are directly comparable.

Statistical analysis

Results are presented as the mean ± s.e.m., with the numbers of rats 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, Inc., Berkeley, CA, USA).

Results

Maternal dexamethasone treatment during pregnancy in the rat leads to intrauterine growth retardation, together with hyperinsulinaemia, hypertension and elevated corticosterone levels in the adult male offspring

We validated the procedure for dexamethasone administration by measurement of fetal weight and blood pressure and plasma analytes in the adult offspring. Maternal dexamethasone administration via an indwelling osmotic pump at a dose of 100 µg/kg body weight per day during the last week of pregnancy did not alter gestation length (control 22.3 ± 0.3 days; dexamethasone 22.8 ± 0.3 days), offspring number (control 12 ± 1; dexamethasone 12 ± 1) or offspring viability (results not shown), but led to a significant 13% (*P*<0.05) decrease in fetal body weight at day 21 of gestation (control 5.4 ± 0.1 g; dexamethasone 4.7 ± 0.1 g). The adult (24-week-old) male offspring of dams treated with dexamethasone (DEX offspring) had developed significant ambient hyperinsulinaemia (80% increase; *P*<0.01) in conjunction with modest but significant increase in systolic hypertension (29% increase; *P*<0.01) and significantly higher plasma corticosterone concentrations (16% increase; *P*<0.05) compared with age-matched controls (CON offspring) (Table 1). Body weight at 24 weeks of age was unaffected by maternal dexamethasone treatment (control 435 ± 10 g; dexamethasone 457 ± 9 g).

Maternal dexamethasone treatment is associated with selective up-regulation of GLUT1 protein expression in hearts of early-growth-retarded adult male offspring

GLUT1 protein expression was analysed by Western blotting in hearts of adult male CON and DEX offspring

Table 1 Maternal dexamethasone (DEX) treatment leads to hyperinsulinaemia, hypertension and elevated corticosterone levels in the adult male offspring. Systolic blood pressures were determined by tail cuff plethysmography at room temperature in lightly anaesthetised rats. Plasma insulin and corticosterone concentrations were measured by radioimmunoassay using commercial kits. Further details are provided in the Materials and Methods section. Data are means ± s.e.m. The number of observations (given in parentheses) indicates the number of individual control or early-growth-retarded adult offspring sampled. Each group was comprised of rats obtained from 3–5 separate litters

	CON	DEX
Plasma insulin (µU/ml)	18 ± 6 (4)	32 ± 6 (4)**
Plasma corticosterone (ng/ml)	296 ± 13 (10)	342 ± 10 (6)*
Systolic blood pressure (mmHg)	80 ± 2 (5)	103 ± 5 (5)**

P*<0.05; *P*<0.01, compared with controls (CON).

at 24 weeks of age (Fig. 1, left hand panel). Direct comparison of adult CON and DEX offspring revealed that maternal dexamethasone treatment led to a marked (2.5-fold) and significant (*P*<0.001) increase in GLUT1 relative protein abundance in the hearts in early-growth-retarded adult (24-week-old) male offspring of dexamethasone-treated dams compared with the age-matched controls. Cardiac GLUT4 protein expression in adulthood was not targeted by dexamethasone-induced early-growth retardation (Fig. 1, right hand panel).

A role for Akt/PKB, but not ERK 1/2, in up-regulation of cardiac GLUT1 protein expression in hearts of early-growth-retarded adult male offspring

We sought to identify the signalling pathways that might be involved in up-regulation of cardiac GLUT1 protein expression in the early-growth-retarded adult male offspring of dexamethasone-treated dams. There was no significant effect of maternal dexamethasone treatment on ERK1 activity (relative abundance: CON, 1.00 ± 0.06; DEX 0.98 ± 0.10) or ERK2 activity (relative abundance: CON, 1.00 ± 0.09; DEX 1.04 ± 0.02), as assessed by Western analysis using phosphorylation-specific antibodies in 24-week-old male offspring. In contrast, using phosphorylation-specific antibodies, we observed a marked and significant 2.2-fold (*P*<0.01) activation of Akt/PKB in the hearts of adult early-growth-retarded male offspring (Fig. 2).

PKC isoform expression and activity is modified in hearts of early-growth-retarded adult male offspring

In the heart, PKC isoforms α, β_{1/2}, δ, ε and ξ have been identified in rat neonatal cardiac myocytes (Bogoyevitch *et al.* 1993, Rybin & Steinberg 1994). In adult rat myocytes and myocardium, PKC isoforms δ and ε seem to

Figure 1 Maternal dexamethasone treatment selectively up-regulates GLUT1 protein expression in hearts of early-growth-retarded adult male offspring. GLUT1 protein expression (left hand panel) and GLUT4 protein expression (right hand panel) were analysed by Western blotting in homogenates of hearts obtained from 24-week-old adult male offspring of control dams (CON; hatched bars) and of dams treated with dexamethasone from day 15 of gestation (DEX; solid bars). Experimental data are presented normalised to the corresponding CON values. Representative immunoblots from gels exposed for a uniform duration are also shown. Data are means $\pm$ S.E.M. for 8 (GLUT1) or 5 (GLUT4) hearts obtained from individual control or early-growth-retarded adult offspring. Each data group was comprised of rats from at least three separate litters. Statistically significant effects of maternal dexamethasone treatment are indicated: *** $P < 0.001$.

be maintained with age, whereas other PKC isoforms may decline (Rybin & Steinberg 1994, Clerk *et al.* 1995). We performed an extensive Western blot survey of PKC expression using protein extracts from hearts of control and early-growth-retarded 24-week-old rats, and identified immunoreactivity for PKC isoforms α , $\beta_{1/2}$, δ , ϵ , and ξ (Fig. 3). Western blot quantification demonstrated significant increases in the classical PKC isoforms PKC β_1 (191%, $P < 0.01$) and PKC β_2 (49%, $P < 0.05$) in hearts of adult early-growth-retarded male offspring. The protein expression of the novel PKC isoform PKC δ was also modestly increased (by 35%; $P < 0.01$); however, that of PKC ϵ and the atypical PKC isoform PKC ξ was unchanged (Fig. 3).

PKC α and PKC ϵ are activated by phosphorylation. The activation state of these PKC isoforms was investigated using phosphorylation specific antibodies (Fig. 4). A significant activation of PKC α (88%, $P < 0.05$) was observed in the hearts of the early-growth-retarded male offspring. We also observed a more subtle, but nonetheless reproducible and significant, 56% ($P < 0.001$) increase in PKC ϵ phosphorylation state in the hearts of the early-growth-retarded rats.

Maternal dexamethasone treatment during pregnancy does not affect the ontogeny of cardiac GLUT1 expression during peri- and early post-natal life

Relative protein expression of GLUT1 in hearts of control rats and rats in the experimental group (maternal dexamethasone treatment from day 15 of gestation) during late fetal life (day 21) and during the first 3 weeks of post-natal life, as quantified by Western blot analysis, are shown in Fig. 5 (left hand panel). Cardiac GLUT1 protein expression in late fetal life was not significantly affected by maternal dexamethasone treatment. Confirming previous studies (Studelska *et al.* 1992, Postic *et al.* 1994), we observed a marked decline in cardiac GLUT1 protein expression during early post-natal development. Investigating the time course of cardiac GLUT1 protein expression down-regulation in detail, we found that cardiac GLUT1 protein expression in the control group was suppressed to a level of 69% ($P < 0.05$) of that of the fetal (day 21) level at post-natal day 7, and continued to decline significantly between post-natal days 7 and 15 (to 46% of the fetal day 21 value; $P < 0.001$). However, by post-natal day 21, cardiac GLUT1 relative abundance in 21-day-old

Figure 2 Akt/PKB activity is increased in hearts of early-growth-retarded adult male offspring. Akt/PKB activation was assessed by Western analysis using phosphorylation-specific antibodies in 24-week-old male offspring. Experimental data are presented normalised to the corresponding CON values. Representative immunoblots from gels exposed for a uniform duration are also shown. Data are means $\pm$ S.E.M. for 6 hearts obtained from individual control (CON) or early-growth-retarded (DEX) adult offspring. Each data group was comprised of rats from at least three separate litters. Statistically significant effects of maternal dexamethasone treatment are indicated: ** $P < 0.01$.

animals was only 39% of that observed in late fetal life ($P < 0.001$), and continued to decline in adulthood (results not shown). As with CON offspring, GLUT1 protein expression was down-regulated during early post-natal development in hearts of DEX offspring at post-natal days 7, 15 and 21 (to 58%, 52% and 52% respectively; $P < 0.001$ at each point). However, no significant differences in cardiac GLUT1 protein expression were observed between CON and DEX offspring at post-natal days 7 and 15, although GLUT1 expression at post-natal day 21 was modestly higher (33%, $P < 0.05$) in hearts of DEX offspring compared with CON offspring.

Post-natal up-regulation of cardiac GLUT4 protein expression is retarded by maternal dexamethasone treatment

The relative levels of GLUT4 protein expression in hearts of control rats at intervals during peri- and early post-natal

Figure 3 PKC isoform protein expression in hearts of early-growth-retarded adult male offspring. Western blot analysis was undertaken using antibodies specific for PKC isoforms α, β1/2, δ, ε, and ζ using protein extracts from hearts of control rats (crosshatched bars) and early-growth-retarded 24-week-old rats (solid bars). Experimental data are presented normalised to the corresponding CON values. Data are means $\pm$ S.E.M. for 6 (PKC isoforms α, β1/2, δ and ε) or 3 (PKC isoform ζ) hearts obtained from individual control or early-growth-retarded adult offspring. Each data group was comprised of rats from at least three separate litters. Statistically significant effects of maternal dexamethasone treatment are indicated: * $P < 0.05$; ** $P < 0.01$.

development, as quantified by Western blot analysis, are shown in Fig. 5 (right hand panel). In the control group, cardiac GLUT4 protein expression was modestly increased at post-natal day 7 (by 45%; not significant (NS)), but subsequently increased dramatically (to 296% of the fetal day 21 value; $P < 0.001$) between post-natal days 7 and 15. There was a further modest rise in GLUT4 protein expression between post-natal days 15 and 21. Thus, cardiac protein expression of GLUT4 increased by approximately 3.4-fold ($P < 0.01$) between fetal day 21 and post-natal day 21. Cardiac GLUT4 expression was subsequently maintained, with little further increase with age (results not shown). Contrasting with the pattern observed in the control group, GLUT4 expression in hearts of DEX offspring tended to be suppressed (by approx. 40% (NS) compared with the late-fetal level) at post-natal day 7. Furthermore, while the cardiac protein expression of GLUT4 increased between post-natal days 7 and 15 (to 200% of the fetal day 21 value; $P < 0.05$) in the DEX offspring, cardiac GLUT4 expression at post-natal day 15 was only approximately 68% of that found in control rats at this stage of development ($P < 0.05$). Thus there appears no necessity for stringent reciprocity of protein expression of GLUT1 and GLUT4 in the early post-natal period for the developing rat heart. Protein expression of GLUT4 in DEX offspring further increased between post-natal days 15 and 21, such that GLUT4 protein expression did not differ significantly between CON and DEX offspring at post-natal day 21. As relative abundance of GLUT4 protein in hearts of offspring of dexamethasone-treated dams did not differ from controls at post-natal day 21, post-natal recovery of cardiac GLUT4 protein expression

Figure 4 Activation state of PKC α and PKC ϵ in hearts of early-growth-retarded adult male offspring. The activation state of PKC α and PKC ϵ was assessed by Western analysis using phosphorylation-specific antibodies in 24-week-old male offspring. Experimental data are presented normalised to the corresponding CON values. Representative immunoblots from gels exposed for a uniform duration are also shown. Data are means $\pm$ S.E.M. for 8 hearts obtained from individual control (CON) or early-growth-retarded (DEX) adult offspring. Each data group was comprised of rats from at least three separate litters. Statistically significant effects of maternal dexamethasone treatment are indicated: * $P < 0.05$; *** $P < 0.001$.

was evident. As in CON offspring, little further change in cardiac GLUT4 protein expression occurred during subsequent development in DEX offspring (results not shown).

Discussion

In the present study, we examined the effect of early-growth retardation induced by maternal dexamethasone treatment during the last third of pregnancy in the rat on cardiac GLUT protein expression in the adult male offspring, and showed that cardiac GLUT1 protein expression is selectively up-regulated. Absence of effects of maternal dexamethasone treatment on cardiac GLUT1 protein expression during early post-natal life suggest that selective up-regulation of cardiac GLUT1 expression in early-growth-retarded male offspring is secondary to effects of early glucocorticoid exposure, possibly through effects to programme the development of hyperinsulinaemia and/or hypertension. Analysis of possible signalling pathways suggests that activation of Akt/PKB (possibly secondary to hyperinsulinaemia) and PKC (possibly secondary to hypertrophic stimulation through hypertension) may mediate the effect.

In the present experiments, cardiac GLUT1 protein expression was up-regulated in hearts of early-growth-restricted male rats, long after exposure to the stimulus (maternal dexamethasone treatment) eliciting growth retardation, suggesting that the early intervention has persistent consequences. Although the modest developmental decline in cardiac GLUT1 expression between post-natal days 15 and 21 was slightly attenuated in offspring of dexamethasone-treated dams, GLUT1 protein expression in hearts of offspring of dexamethasone-treated dams was comparable to that of control offspring during the first 2 weeks of life. This finding suggests that dexamethasone *per se* is unimportant in mediating this effect, and thus increased cardiac GLUT1 expression is an indirect effect of 'programming' of other parameters. Hypertension, as is observed in early-growth-retarded rats in the present study, is an established stimulus for cardiac hypertrophy, and previous studies have reported increased GLUT1 mRNA and GLUT1 protein expression in a canine model of persistent myocardial ischaemia (Brosius *et al.* 1997). Conversely, cardiac GLUT1 protein expression falls during fasting, when insulin levels are low, and maintaining insulin levels (by insulin infusion at euglycaemia) during fasting prevents the decline in GLUT1 (Laybutt *et al.* 1997): insulin levels are elevated in

Figure 5 Ontogeny of cardiac GLUT1 and GLUT4 protein expression during peri- and early post-natal life. GLUT1 and GLUT4 protein expression was determined by Western blot analysis of homogenates of hearts obtained from rats at approximately weekly intervals during the first three weeks of post-natal development (7 days, N7; 15 days, N15; and 21 days, N21). Relative protein expression of GLUT1 in hearts of control (CON) rats (open symbols) and rats in the experimental group (maternal dexamethasone treatment from day 15 of gestation) (closed symbols) are shown in the left hand panel. The relative levels of GLUT4 protein expression in hearts of control (open symbols) and experimental rats (closed symbols) at intervals during peri- and early post-natal development, as quantified by Western blot analysis, are shown in the right hand panel. Data are presented relative to the CON values in 21-day-old fetuses (F21) together with representative immunoblots (control, C; dexamethasone-treated, D). Data are means $\pm$ S.E.M. for pooled hearts obtained from individual litters of control dams (3) or dexamethasone-treated dams (6). GLUT1 protein expression was down-regulated during early post-natal development in hearts of CON offspring at post-natal days 7 ($P < 0.05$), 15 ($P < 0.001$) and 21 ($P < 0.001$) and in DEX offspring at post-natal days 7, 15 and 21 ($P < 0.001$ at each point). GLUT4 protein expression was up-regulated during early post-natal development in hearts of CON offspring at post-natal days 15 ($P < 0.05$) and 21 ($P < 0.05$) and in DEX offspring at post-natal days 15 and 21 ($P < 0.05$ at each point). Statistically significant effects of maternal dexamethasone treatment are indicated: * $P < 0.05$.

early-growth-retarded rats in the present study. Thus, either hypertension or hyperinsulinaemia might underlie increased cardiac GLUT1 expression.

Insight into which of these possible signals might be instrumental in this effect was gained from analysis of reactive signalling pathways. Insulin binds to a cell surface receptor and activates an intrinsic receptor tyrosine kinase, a process that ultimately stimulates two major signalling cascades (Shepherd *et al.* 1998). One cascade leads to the activation of MAPK and the phosphorylation of transcrip-

tion factors (Robinson & Cobb 1997). The second cascade involves phosphatidylinositol-3 kinase (Toker & Cantley 1997) and numerous potential downstream effectors, including the serine/threonine kinase Akt/PKB (Coffer *et al.* 1998). In the present study, we observed that increased GLUT1 protein expression in hearts of early-growth-retarded adult male rats was associated with significant activation of Akt/PKB in the heart, as assessed from increased relative abundance of phospho-Akt/PKB. Akt/PKB activity reflects contributions from three distinct

isozymes (Akt1–3), all of which are regulated primarily by phosphorylation (Coffer *et al.* 1998). Akt1/PKB α is the predominant isoform in most tissues, whereas Akt3/PKB γ shows a restricted pattern of expression. The highest expression of Akt2/PKB β is observed in insulin-responsive tissues, including the heart. Although data as to whether Akt/PKB can mediate transcriptional responses to insulin are conflicting (see e.g. O'Brien & Granner 1996, Agati *et al.* 1998, Cichy *et al.* 1998, Dickens *et al.* 1998, Liao *et al.* 1998, Wang & Sul 1998), Barthel *et al.* (1999) found that Akt/PKB can induce GLUT1 gene transcription in mouse hepatoma cells, activation of the Akt1 kinase suffices to induce GLUT1 transcription and GLUT1 protein accumulation to an extent similar to that induced by insulin, and expression of an inactive Akt1 partially inhibits the ability of insulin to stimulate GLUT1 gene expression. It is therefore concluded that an increase in Akt/PKB activity may be an important event in the signalling pathway through which insulin may regulate GLUT1 expression in the adult heart. As well as influencing glucose metabolism, evidence is mounting that one of the major functions of Akt/PKB is to promote cell survival and to block apoptosis. Although downstream cellular processes regulated by Akt/PKB in the present study were not examined, our data also imply that increased Akt/PKB may confer resistance to apoptosis in hearts of early-growth-retarded offspring.

Studies, mainly with neonatal rat myocytes, demonstrate associations between hypertrophic stimulation and all three main branches of the MAPK signalling cascade, namely ERKs, Jun-N-terminal kinases (JNKs) and p38s (Sadoshima & Izumo 1993a, Bogoyevitch *et al.* 1994, Thorburn *et al.* 1994a,b, Post *et al.* 1996, Ramirez *et al.* 1997, Zechner *et al.* 1997, Choukroun *et al.* 1998, 1999, Nemoto *et al.* 1998, Wang *et al.* 1998a,b, Hines *et al.* 1999, reviewed by Sugden 1999). The ERKs also induce GLUT1 expression at the level of transcription (Montessuit & Thorburn 1999) and are thought to be anti-apoptotic, whereas activation of JNK and p38 MAPKs is generally associated with promotion of apoptosis. In the present study of adult rat hearts, we found no evidence for activation of ERK 1 and 2, and in the light of this negative finding together with that of activation of Akt/PKB, we did not go on to investigate whether the other two main branches of the MAPK signalling cascade (JNKs and p38s), were similarly unaffected.

The protein expression of most PKC isoforms is down-regulated in the heart after birth (Bogoyevitch *et al.* 1993, Rybin & Steinberg 1994, Clerk *et al.* 1995). Hypertrophic stimulation of the adult heart is accompanied by recapitulation of the expression of certain fetal genes. In the present experiments, Western blot quantification demonstrated selective increases in protein expression of both classical and novel PKC isoforms in hearts of early-growth-retarded adult rats. In that the fetal PKC isoform expression programme is not globally reiterated, these data indicate

that the cardiac fetal genes for individual PKC isoforms are regulated independently. Within this context, it is of interest that in our experiments the fetal programme is recapitulated with respect to up-regulation of cardiac GLUT1 protein expression, but not with respect to GLUT4 protein expression (i.e. GLUT4 protein expression remains high despite up-regulation of GLUT1). Thus, available data suggest that the mechanisms or regulation of cardiac GLUT1 expression observed in adulthood in our model of early-growth retardation are distinct from those responsible for modulating cardiac GLUT4 protein expression. In support of independent regulation of GLUT1 and GLUT4 protein expression in the adult heart, pressure-overload cardiac hypertrophy induced by aortic banding is associated with down-regulation of GLUT4 protein expression in the absence of any change in GLUT1 expression (Depre *et al.* 1998).

We identified modest but significant activation of PKC α and ϵ by phosphorylation, together with significant up-regulation of cardiac PKC $\beta_{1/2}$ and PKC δ protein expression in hearts of the early-growth-retarded male rats. PKC α and ϵ have been reported previously to be up-regulated in pressure-overload hypertrophy in the rat (Paul *et al.* 1997, Takeishi *et al.* 1999). The induction of apoptosis by ceramide involves the dephosphorylation and inhibition of PKC α (Lee *et al.* 1996), whereas a reduction in the level of PKC α expression results in apoptosis in a number of cell types (Haimovitz-Friedman *et al.* 1994, Dooley *et al.* 1998, Whelan & Parker 1998). Thus, the increase in PKC α activity observed in the hearts of early-growth-restricted male rats in the present experiments both mimics that observed in pressure-overload hypertrophy and would be predicted to suppress cardiac myocyte apoptosis. PKC ϵ signalling is important for normal post-natal maturation of myocardial development (Bogoyevitch *et al.* 1993, Rybin & Steinberg 1994) and the suggestion has been made that activation of PKC ϵ could be of potential therapeutic value for improving cardiac growth and survival after ischaemic insult (Mochly-Rosen *et al.* 2000, for review see Naruse & King 2000). Thus, activation of PKC ϵ in hearts of early-growth-retarded rats would be predicted to confer protection against ischaemia. By contrast, most studies seem to suggest that up-regulation of PKC $\beta_{1/2}$ may have adverse consequences, including decrease in cardiac contractility (Bowman *et al.* 1997, Takeishi *et al.* 1998) and PKC δ is activated *in vivo* by diabetes and ischaemia (Kawamura *et al.* 1998, Koya & King 1998): we demonstrate up-regulation of the protein expression of all of these PKC isoforms in the hearts of early-growth-retarded offspring. Thus, overall, our results suggest that early-growth retardation induced by maternal dexamethasone treatment may evoke mechanisms to protect cardiac function that may, at least in part, attempt to counter adverse effects – perhaps secondary to programming of hypertension and/or

abnormal glucose metabolism – that may ultimately lead to heart failure.

In conclusion, our data demonstrate up-regulation of cardiac GLUT1 protein expression in early-growth-retarded, hypertensive, hyperinsulinaemic adult offspring of dexamethasone-treated dams, and suggest that GLUT1 induction may result from altered signal transduction pathways including those involving Akt/PKB in association with activation of one or more anti-apoptotic PKC isoforms. Although activation of the MAPK pathways may be involved in signalling hypertrophic stimulation in neonatal myocytes, we found no evidence that sustained activation of ERK1 and ERK2 is obligatory for up-regulation of cardiac GLUT1 expression in adulthood.

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