Cortisol influences the ontogeny of both α- and β-subunits of the cardiac sodium channel in fetal sheep

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Abstract

During development, the heart has to adapt to changes in shape, size and, at birth, to significant changes in arterial pressure. The orderly contraction of the heart is dependent on the coordinated expression of ion channels at appropriate densities in individual cardiac myocytes. The present study demonstrated that the expression of the α-subunit of the cardiac sodium channel, SCN5a, was high at mid gestation but then decreased until 10 days before birth before increasing again. Whereas the β-subunit, SCN1b, gradually increased in expression towards partum, there was no detectable expression of SCN3b at any gestational time point. Fetal adrenalectomy prior to the normal prepartum surge in cortisol caused a reduction in expression of SCN1b and a 7·0 kb transcript of SCN5a, but not the major 8·5 kb transcript. Conversely, cortisol infusion into immature fetuses precociously increased expression levels of SCN1b and the SCN5a 7·0 kb transcript. The results show that cortisol regulates cardiac SCN gene expression in fetal sheep during late gestation. These findings could have implications for the aetiology of sudden infant death syndrome and for the intrauterine programming of adult cardiovascular disease.


Introduction

In all species studied to date, there are changes in cardiac function at birth that are essential for the transition to extrauterine life (Liggins 1994, Fowden et al. 1998). The heart has to adapt to the increased perfusion of the neonatal lungs and pump against a greater pressure. In many tissues, preparation for the postnatal adaptations vital for neonatal survival has been shown to begin during late gestation and to depend on increased cortisol (glucocorticoid) secretion by the fetal adrenal glands. Furthermore, these changes have been demonstrated in a number of species including rodents, domestic animals, and human and non-human primates (Liggins 1994).

The heart maintains its pumping function through an electrical activity that is governed by the integrated behaviour of a wide range of ion channels expressed in cardiac myocytes. The voltage-gated sodium channels are critical elements of action potential initiation and propagation in excitable cells. They are multimeric structures composed of a pore-forming α-subunit and smaller auxiliary subunits embedded in the plasma membrane. The pore-forming subunits of cardiac sodium channels are encoded by SCN5a. Three sodium channel auxiliary subunit genes have been defined thus far: SCN1b (encodes β1 and β1A), SCN2b (encodes β2) and SCN3b (encodes β3) (Catterall 2000). Both SCN1b and SCN3b β-subunits are expressed in cardiac tissue from a range of species (including rodents, humans and sheep) and are known to modulate the kinetics of the cardiac sodium channel α-subunit (Nuss et al. 1995, Qu et al. 1995, Dhar Malhotra et al. 2001, Fahmi et al. 2001). Nevertheless, the role of β-subunits in the heart is incompletely understood, in particular there is very little known about what regulates the expression of sodium channel subunits in the heart.

Recent reports in man have linked mutations in SCN5a with sudden infant death syndrome (SIDS) (Schwartz et al. 2000, Ackerman et al. 2001, Wedekind et al. 2001). The peak incidence of SIDS is between 2 and 4 months, which corresponds to the period when the normal circadian rhythm in cortisol levels is being established in the human infant (Gordon et al. 1999). Further, cohort analysis using data from the 1987 United States Birth Cohort Linked Birth/Infant Death implied that preterm human infants are at higher risks of SIDS than term infants (Malloy & Hoffman 1995), thus raising the possibility that abnormalities in SCN expression linked to dysregulation of
cortisol metabolism may contribute to the cause of death in SIDS in human infants.

The aims of this study therefore were to investigate the patterns of expression of SCN5a, SCN1b and SCN3b in the fetal sheep heart during development and to examine the role of cortisol in regulating developmental changes in sodium channel subunit expression.

Materials and Methods

Animals

A total of 30 Welsh Mountain sheep fetuses of known gestational age were used in this study together with two additional newborn lambs and three adult pregnant ewes. The ewes were housed in individual cages and fed with concentrate (200 g/day: H.G. Beart, Stonebridge, Kings Lynn, UK) and hay and allowed to feed ad libitum. Food but not water was withheld for 18–24 h before surgery. Normal feeding patterns were restored within 24 h of operation. All experimental procedures were carried out in accordance with the UK Animals (Scientific Procedures) Act 1986.

Surgical and experimental procedures

Under halothane anaesthesia (1.5% in O2/N2O) one of the following procedures was carried out between 115 and 118 days of gestation (term=143 ± 2 days) using previously published surgical methods (Li et al. 1996): (a) vascular catheterization of intact control fetuses (n=12) or (b) bilateral adrenalectomy of the fetus (n=5). At least 6 days after catheterization, the intact fetuses were infused intravenously with either cortisol (1–3 mg/day in 3 ml 0.9% (wt/vol) saline, EF-Cortelan, Glaxo Ltd, Greenford, Middlesex, UK, n=6) or saline (0.9% wt/vol at 3 ml/day, n=6) for 5 days before delivery for tissue collection at 125–130 days of gestation. The dose of cortisol was chosen to provide a progressive rise in the fetal plasma cortisol concentration that mimicked the normal prepartum cortisol surge (Fowden et al. 1996).

Ovine tissue collection

All infused (n=12), adrenalectomized fetuses (n=5 at 139–144 days) and 13 additional untreated intact fetuses (104–145 days) were delivered by Caesarean section under sodium pentobarbitone anaesthesia (20 mg/kg i.v.). Cord blood samples were taken from the majority of fetuses by venepuncture from the umbilical artery after anaesthesia had been induced. After administration of a lethal dose of sodium pentobarbitone (200 mg/kg), 3–5 g samples of the ventricle tips of the fetal heart were collected and immediately frozen in liquid nitrogen and stored at −80 °C. A range of other tissues was also collected as part of another study. All blood samples were centrifuged immediately at 4 °C and the plasma stored at −20 °C until analysis for plasma cortisol. Plasma cortisol concentrations were measured by radioimmunoassay validated for use with ovine plasma (Robinson et al. 1983). At delivery, no obvious adrenal remnants were found in any adrenalectomized fetuses.

Molecular analyses

Northern and Western blot analyses were performed on the tissue within 6 months of collection as described previously (Fahmi et al. 2001). Sheep cDNA probes (see Table 1 for the list of forward and reverse primers used for cDNA synthesis) for SCN5a, SCN1b, SCN3b and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were labelled with 32P-CTP using the multiprime labelling kit (Amersham). Membrane proteins were extracted from three fetal sheep hearts for each gestational period tested. Proteins levels were quantified using standard Bradford assay (BioRad, UK) and 60 µg of protein were separated on a 3–8% Tris–glycine SDS–polyacrylamide gels and transferred to polyvinylidene difluoride membranes. The SCN1b rabbit polyclonal antibody (a kind gift from Lori Isom, University of Michigan, MI, USA) was raised against the peptide sequence corresponding to the initial 18 amino acids of rat β1 sequence, GGCVEVDSETEAVYGMTF (Isom et al. 1992). The SCN3b rabbit polyclonal antibody was raised against the peptide TEEQKYYNAMKKLGSK(C), corresponding to residues 1491–1508 of the α-subunit of rat type I voltage gate sodium channel.

Data analysis

Autoradiograms were scanned (256 greyscales) using a UMAX Powerlook III scanner and the optical density

<table>
<thead>
<tr>
<th>Table 1</th>
<th>PCR primers used for synthesis of cDNA probes for Northern blotting</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCN5a (exon 15–16)</td>
<td>F CCT CGA CCC CTA CTA CTA CTT CC</td>
</tr>
<tr>
<td>R GAA GAG ATT CAG GAC CAA AGG</td>
<td></td>
</tr>
<tr>
<td>SCN5a (exon 20/21)</td>
<td>F GGC TGT GTC CGG CGG TGT CC</td>
</tr>
<tr>
<td>R TTG AGC AGC ATC TCC AGC AC</td>
<td></td>
</tr>
<tr>
<td>SCN1b</td>
<td>F GAT CCT GGC CAA TGG CAG CCG G</td>
</tr>
<tr>
<td>R CGA TGG ATG CCA TGT CTC TGT TG</td>
<td></td>
</tr>
<tr>
<td>SCN3b</td>
<td>F GTG TGT GTG GAA GTG CCC TCG GAG</td>
</tr>
<tr>
<td>R GAC CAC CGA GGT GAA GTC TCC TCC</td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>F GAC TCA TGA CCA CAG TCC ATG C</td>
</tr>
<tr>
<td>R CCT GTT GCT GTA GCC AAA TTC G</td>
<td></td>
</tr>
</tbody>
</table>

F, forward; R, reverse.
(OD) for each autoradiograph was calibrated using a photographic step tablet (Eastman Kodak Company, Rochester, NY, USA). All results are reported as means ± S.E.M. Statistical significance was assessed by ANOVA, Student–Newman–Keuls t-tests, or linear regressions using Statview v5·0·1. (SAS Institute, Inc., Cary, NC, USA). A P value of < 0·05 was regarded as significant.

Results

Developmental changes

Typical examples of Northern blots illustrating the developmental changes in sodium channel subunit mRNA expression in sheep heart are shown in Fig. 1. There is a relatively high level of expression of SCN5a at 104–113 days of gestation, which then declined between 126 and 137 days of gestation before increasing again during late gestation (139–141 days). The SCN1b transcript was barely detectable at 104–113 days of gestation but thereafter there was a steady increase in the level of expression (Fig. 1). The rise in SCN1b expression close to term closely paralleled the normal prepartum rise in fetal plasma cortisol (Table 2). The SCN3b transcript was undetectable in hearts prior to birth (Fig. 1), although it was clearly seen in the left ventricle samples from adult animals (Fig. 1, right panel).

Table 2 Plasma cortisol levels and changes in relative abundances of SCN5a and SCN1b mRNA during development in fetal sheep. n=3 at each gestational age range

<table>
<thead>
<tr>
<th>Gestational age (days)</th>
<th>110–113</th>
<th>127–132</th>
<th>137–141</th>
<th>143 to NB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cortisol (ng/ml)</td>
<td>7·7 ± 2·3a</td>
<td>10·9 ± 2·1a</td>
<td>33·8 ± 6·8b</td>
<td>54·8 ± 12·8c</td>
</tr>
<tr>
<td>SCN5a (OD)</td>
<td>0·31 ± 0·008a</td>
<td>0·14 ± 0·04b</td>
<td>0·30 ± 0·10a</td>
<td>0·45 ± 0·12a</td>
</tr>
<tr>
<td>SCN1b (OD)</td>
<td>0·035 ± 0·01a</td>
<td>0·071 ± 0·01b</td>
<td>0·15 ± 0·04c</td>
<td>0·18 ± 0·05c</td>
</tr>
</tbody>
</table>

Values within each row with different superscripts are significantly different from each other (ANOVA, P<0·05). OD, optical density; NB, newborn.
throughout gestation (OD at 104–113 days of gestation 0.68 ± 0.09, n = 3; at 128–139 days of gestation 0.66 ± 0.11, n = 3; and in day 141 and newborn 0.69 ± 0.11, n = 3). A minor band at > 50 kDa was also detected in newborn sheep and late gestation fetal hearts. SCN3b antibody did not detect any protein at any of the gestational ages examined (data not shown).

The effect of manipulating the cortisol level

To determine whether changes in sodium channel subunit expression during development were related to changes in plasma cortisol levels, sodium channel mRNA and protein expression were measured in hearts taken from fetuses near term, in which the prepartum rise in plasma cortisol had been prevented by adrenalectomy and from 125–130 days of gestation fetuses that had been infused with cortisol for 5 days before delivery to mimic the prepartum cortisol surge (Table 3).

Typical examples of Northern and Western blots performed on tissues following alteration of cortisol levels are illustrated in Fig. 3 and the results are summarized in Table 3. Increases in the fetal cortisol level had little effect on the level of expression of the 8.5 kb SCN5a transcript (Fig. 3A,B). However, expression of a 7.0 kb SCN5a transcript (see below) was affected by changes in the fetal cortisol level (Fig. 3A,B). An increase in fetal cortisol concentration, either endogenously or by exogenous infusion, was associated with a significant increase in expression of the 7.0 kb transcript (see Table 3). Conversely, when the normal prepartum rise in cortisol was prevented by fetal adrenalectomy, the increase in expression of the 7.0 kb transcript was prevented (see Fig. 3B and Table 3). Western blots using SP19 showed no significant effect of cortisol on levels of sodium channel protein (Fig. 3C).

Expression of both SCN1b mRNA and protein were affected by the fetal cortisol level. Cortisol infusion increased SCN1b mRNA and protein expression in fetal hearts at 125–130 days (Fig. 3A,C) while adrenalectomy prevented the normal increase in SCN1b mRNA and protein levels observed in late gestation (Fig. 3B,D, Table 3). Furthermore, there was a significant correlation between the level of SCN1b mRNA and plasma cortisol levels (see Fig. 4).

Discussion

Sodium channel subunit expression during heart development

The present study demonstrates that there is a heterogeneous expression of the sodium channel, SCN5a and its auxiliary α-subunits during heart development in sheep. SCN5a mRNA and protein expression are high at 70% gestation (~110 days of gestation), before declining and then rising again 10–15 days prepartum (see Fig. 1). Expression levels of SCN1b mRNA relative to GAPDH mRNA were low at 104–113 days of gestation and increased gradually until birth. On the other hand, SCN3b mRNA and protein were not detected at any stage of heart development, although it was highly expressed in sheep adult left ventricle (see Fig. 1). The increase in SCN1b mRNA and immunoreactive protein during late gestation would be expected to lead to

Table 3 Plasma cortisol levels and changes in relative abundances of SCN5a and SCN1b mRNA in fetal sheep following manipulation of cortisol levels. n = 3 at each gestational age range

<table>
<thead>
<tr>
<th>Gestational age</th>
<th>125–130 days</th>
<th>143–145 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>Saline</td>
<td>Cortisol</td>
</tr>
<tr>
<td>Cortisol (ng/ml)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SCN5a (OD), 8.5 kb</td>
<td>10.9 ± 2.1a</td>
<td>52.3 ± 7.2b</td>
</tr>
<tr>
<td>SCN5a (OD), 7.0 kb</td>
<td>0.12 ± 0.04</td>
<td>0.12 ± 0.04</td>
</tr>
<tr>
<td>SCN1b (OD)</td>
<td>0.05 ± 0.001a</td>
<td>0.131 ± 0.04b</td>
</tr>
<tr>
<td>SCN1b (OD)</td>
<td>0.08 ± 0.001a</td>
<td>0.187 ± 0.03b</td>
</tr>
</tbody>
</table>

Values with different superscripts within each row for each group (i.e. 125–130 or 143–145 days of gestation) are significantly different from each other (ANOVA, P < 0.05).
an increase in the density of functional sodium channels (Fahmi et al. 2001). Therefore, the changes in sodium channel expression reported here are consistent with the ontogenesis of the electrical properties of myocardial cells during development in mice, guinea pigs and chick embryos (Renaud et al. 1981, Davies et al. 1996, Kato et al. 1996). At the early stages of embryonic development the maximal upstroke velocity ($V_{\text{max}}$) of the action potential is clearly sodium channel dependent, although it is significantly slower than in adults ($V_{\text{max}} < 10$ V/s in embryos compared with $\sim 100$ V/s in neonates and adults) (Renaud et al. 1981). Further, sodium current amplitude is low in myocytes isolated from murine hearts at mid gestation ($\sim 10$ pA/pF), but increases by birth in mice to $\sim 100$ pA/pF (Davies et al. 1996). Similarly, in myocytes from guinea pigs, there is a shortening of the action potential during the period of late gestation during which fetal cortisol concentrations rise (Kato et al. 1996, Fowden et al. 1998). The significant level of SCN5a expression in ovine hearts at mid gestation in the current study is also consistent with the recent finding that mice with homozygous SCN5a knockout die during mid gestation (approx. day 10.5) (Papadatos et al. 2002).

The changes in sodium channel subunit density levels during development are also likely to contribute to the slower conduction in fetal compared with adult hearts. The expression of gap junctional proteins in cardiac cells will also influence conduction velocity during fetal development. Connexins 40 and 43 mRNA levels are low at early gestation and then increase towards term in rat and mouse (Fishman et al. 1991, Delorme et al. 1997). It is therefore likely that increases in conduction velocity are a reflection of changes in both sodium channel density and gap junction density.
Effect of cortisol on expression levels of sodium channel

One of the key changes that govern maturation of the fetal tissues is the prepartum surge in hormone release, particularly cortisol secretion by the fetal adrenal glands (Liggins 1994). Cortisol has been implicated in the regulation of many ion channels. For example, dexamethasone, a glucocorticoid receptor agonist, upregulates the expression of Kv1.5 potassium channel mRNA and immunoreactive protein in rat ventricles (Takimoto & Levitan 1994). Cortisol also mediates the developmental changes in expression of ion channels and transporters in other tissues including, for example, the rise in both Na+/K+-ATPase mRNA abundance (Celsi et al. 1993) and the Na+/H+ exchanger activity and mRNA levels (Guillery et al. 1995) in the fetal ovine kidney.

The present results demonstrate that cortisol specifically upregulates the expression of SCN1b (see Figs 1 and 4) and the 7·0 kb transcript of SCN5a, but not the 8·5 kb transcript of SCN5a in fetal ovine heart in vivo. Expression of SCN1b and the 7·0 kb transcript of SCN5a increased towards term in parallel with the prepartum cortisol surge in the fetus. When this increase in cortisol was prevented by fetal adrenalectomy, the prepartum increases in SCN1b and SCN5a (7·0 kb transcript) were abolished, whilst levels of SCN5a (8·5 kb transcript) were similar to those observed in intact fetuses close to term (see Fig. 3). Administration of physiological doses of cortisol to intact fetuses at times in mid gestation (125–130 days) also increased the levels of SCN1b and SCN5a (7·0 kb transcript) to values similar to those observed in older fetuses with high endogenous cortisol concentrations (Derks et al. 1997, Fowden et al. 1998). SCN5a (8·5 kb transcript) levels did not increase as a result of cortisol infusion. The simplest explanation for the results observed in this study is a direct effect of cortisol, acting via glucocorticoid response elements, GREs (Rousseau 1984). Several of the genes known to be regulated by cortisol (e.g. insulin-like growth factor-II (IGF-II) and erythropoietin) have GREs in their promoter regions to allow direct transcriptional control of the gene by cortisol in fetal sheep (Li et al. 1996, Lim et al. 1996). However, other genes that are apparently glucocorticoid sensitive do not appear to have recognizable GRE consensus sequences (Dickson et al. 1991). Alternatively, cortisol may act indirectly through other hormones such as insulin–like growth factor-I (IGF-I) and triiodothyronine (T₃), and/or enzymes, such as the S6K1 kinase, which are glucocorticoid inducible and known to affect cardiac function postnatally (Li et al. 1996, Wickenden et al. 1997, Bohmer et al. 2003). Clearly, further studies are required to determine the mechanism by which cortisol acts on the various promoters of the SCN1b and SCN5a genes.

In summary, the current results demonstrate that cortisol has a physiological role in regulating the cardiac SCN channel and its subunits during ovine development. Abnormalities in SCN gene expression may, therefore, occur in infants delivered prematurely before the prepartum cortisol surge. Similarly, early exposure to cortisol during adverse intrauterine conditions may lead to inappropriate changes in SCN gene expression with long-term consequences for cardiac function, both before and after birth. These findings, therefore, have important implications both for the aetiology of SIDS and for the intrauterine programming of adult cardiovascular disease (Barker 2002).

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