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Loss of the Normal Epicardial to Endocardial Gradient of cftr mRNA Expression in the Hypertrophied Rabbit Left Ventricle

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The electrical instability of hypertrophied and failing hearts is caused by delayed repolarisation, which is thought to be due in part to altered levels and/or patterns of expression of ion channel genes. The aim of this study was to investigate changes in the levels and pattern of cystic fibrosis transmembrane conductance regulator (cftr) mRNA expression in a combined pressure and volume overload model of heart failure in the rabbit, using *in situ* mRNA hybridisation. There was a decrease in cftr mRNA expression, primarily due to a decrease in epicardial expression and, hence, loss of the normal epicardial to endocardial gradient of cftr mRNA expression in the rabbit left ventricle. In contrast there was an increase in atrial natriuretic factor (anf) mRNA expression in the hypertrophied hearts with preferential reexpression in subendocardial regions. The patterns of both cftr and anf mRNA expression in the hypertrophied hearts were similar to those seen in embryonic hearts. This suggests that the reversion to an embryonic pattern of gene expression in cardiac hypertrophy applies to ion channel genes. The loss of the normal transmural gradient of repolarising ion channels is likely to contribute to instability of repolarisation in the hypertrophied heart and hence increased risk of cardiac arrhythmias in patients with heart failure.

Key Words: **mRNA *in situ* hybridisation; CFTR; ANF; cardiac hypertrophy; heart failure; embryonic.**

Heart failure is a major cause of morbidity and mortality [1]. At least 50% of deaths in patients with heart failure are due to ventricular arrhythmias [2]. Arrhythmias represent the end product of abnormal ion-channel expression or function [3], e.g., mutations in ion channels account for the increased risk of arrhythmias in the Long QT syndrome [4]. The increased risk of arrhythmias in patients with heart failure is thought to be due, at least in part, to delayed repolarisation caused by decreases in the levels of expression of repolarising ion channels [5].

Altered levels of activity of multiple ion channels and transporters have been reported both in human heart failure and in animal models of heart failure. These include decreases in transient outward (I_{TO}) K^+ currents [6–9], inward rectifying K^+ currents [6, 8], delayed rectifier K^+ currents [10], and cAMP-activated chloride currents [11] and an increase in Na^+–Ca^{2+} exchanger (NCX) activity [12]. In addition to decreases in repolarising ion currents there also appears to be changes in their patterns of distribution. For example, Shipsey *et al.* [13] demonstrated that there was a more marked decrease in I_{TO} activity in subepicardial myocytes compared to subendocardial myocytes in a catecholamine-induced model of hypertrophy in the rat. Consequently there was a loss of the normal epicardial to endocardial gradient of I_{TO} activity and this, they suggested, could contribute to T-wave inversion in the electrocardiogram, a common finding in ventricular hypertrophy.

It has often been postulated that cardiac hypertrophy is characterised by reexpression of an embryonic pattern of gene expression [14]. For example, the atrial natriuretic factor (anf) gene, which is expressed in both atria and ventricles in embryonic hearts but is restricted to the atria in the normal adult heart, is reexpressed in the ventricles of hypertrophied hearts [15]. The patterns and levels of ion channel gene expression also change during development. However, there are relatively few studies that have investigated whether changes in ion channel activity in hypertrophied hearts

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are due to decreases in the level of ion channel mRNA expression [9, 16–18] and there are no studies that have tested whether the changes in ion channel mRNA expression in hypertrophied hearts represents a reversion to embryonic levels and patterns of expression. Recently, we have shown that the cystic fibrosis transmembrane conductance regulator (cftr) mRNA, which encodes for the cAMP-activated chloride conductance, is developmentally regulated in the rabbit left ventricle [19]. Prior to birth there is no gradient of cftr mRNA expression across the rabbit left ventricle but within 6 weeks of birth the normal adult gradient of cftr mRNA (epicardial:endocardial ~2.5:1) has developed [19]. The aim of this study, therefore, was to investigate whether there was a reversion to the embryonic level and pattern of cftr mRNA expression following the induction of heart failure in the rabbit.

MATERIALS AND METHODS

Induction of heart failure. Heart failure was produced by combined volume and pressure overload as previously described [20]. NZ White rabbits were anaesthetised with thiopental sodium (3.4 ± 0.3 mL, 50 mg/mL i.v.). Volume overload was produced by partial destruction of an aortic valve leaflet using a bevelled polyethylene catheter connected to a pressure transducer introduced via the carotid artery. The catheter was repeatedly passed through the aortic valve until the pulse pressure increased by 50%. Two weeks later, using a similar anaesthetic regimen, pressure overload was induced by constricting the abdominal aorta, immediately below the diaphragm, by tying a ligature around the aorta against a polyethylene catheter (2.42 mm o.d.) which was then withdrawn. Sham operated animals underwent identical procedures except without aortic valve destruction and abdominal aortic ligature. In total 5 animals underwent the experimental protocol and 5 underwent the sham operation. Two weeks later, animals were killed with an overdose of thiopental sodium (200 mg/kg i.v.). Hearts were rapidly excised and then retrogradely perfused with phosphate-buffered saline (4°C) containing heparin (4 U/ml) followed by perfusion with 4% paraformaldehyde solution. Hearts were stored in 4% paraformaldehyde solution containing 10% sucrose, at 4°C for at least 48 h before sectioning.

mRNA in situ hybridization. In situ hybridization experiments were carried out essentially as previously described [21–23]. Briefly, 10-μm frozen sections of the left ventricular free wall (LVFW) were digested with proteinase K then hybridised overnight at 50°C with sense or antisense ^{35}S-UTP-labeled riboprobes for anf, cftr or gapdh (see below). Sections were then digested with RNase A and washed in 15 mM NaCl, 1.5 mM Na-citrate at 60°C to remove unhybridised probe. X-ray film contact sheets (Biomax MR-1; Kodak, Rochester NY) were exposed to hybridised slides for 48 h (cftr and anf probes) or 5 h (gapdh probes) then developed and fixed. Autoradiograms were scanned (256 grey scales) at a resolution of 600 dpi using a UMAX Powerlook III scanner. The optical density for each autoradiograph was calibrated using a photographic step tablet (Eastman Kodak Co., Rochester, NY). Scanned images were analysed using NIH image software (public domain software). False color images were obtained using a linear transformation.

Riboprobes. Riboprobes for rabbit cardiac cftr and gapdh were the same as previously described [23]. A 391-bp fragment of the rabbit anf cDNA was prepared by PCR amplification of adult rabbit atrium cDNA using the following primers; 5′ ctcttctgtctggcattctgg 3′ and 5′ caatcctgtcgatcctgcc 3′. We used a touch-down PCR protocol: denaturation for 1 min at 95°C, annealing for 1 min at 65°C (2 cycles), 60°C (4 cycles), 56°C (7 cycles), 52°C (10 cycles), 47°C (15 cycles), and extension at 72°C for 1.5 min. cDNA Fragments were ligated into the pGEM-T vector and the identity and orientation were confirmed by sequencing using both the T7 and SP6 promoters of the pGEM-T vector (Promega, Southampton, UK). Single-stranded, ^{35}S-labeled RNA probes were synthesised by linearising vectors containing the cDNA inserts with the use of appropriate restriction enzymes followed by *in vitro* runoff RNA transcription using the appropriate RNA polymerase (SP6, T3 or T7 RNA polymerase, Epicentre Technologies). There was no cold UTP present in the labeling reaction, so all probes had approximately the same specific activity. Labeled probes were separated from unincorporated label by Sephadex G50 chromatography, and probes were diluted into the hybridisation mixture so that the final concentration of all probes was 2–3 × 10^7 counts min^{-1} mL^{-1}.

RESULTS

Examples of *in situ* mRNA hybridization autoradiograms from sham-operated and hypertrophied hearts are shown in Fig. 1. There is a marked increase in expression of anf mRNA and a considerable downregulation of cftr mRNA expression. Furthermore, there is a loss of the normal epicardial to endocardial gradient of cftr mRNA expression and the marked increase in anf mRNA expression is predominantly seen in the subendocardial regions. In contrast there are no significant changes in gapdh mRNA expression between sham-operated and hypertrophy animals.

For comparison, examples of *in situ* mRNA hybridization autoradiograms from embryonic rabbit hearts (day 30/31 day gestation) are shown in Fig. 2. The expression of anf mRNA is almost exclusively located to the inner layers of the endocardium (in both the left and right ventricles) and cftr mRNA is uniformly expressed throughout the myocardium in the embryonic hearts. These distributions of cftr and anf mRNA in embryonic hearts are very similar to that observed for the hypertrophied adult hearts.

The level of increase in anf mRNA expression in hypertrophied hearts varied from animal to animal and it was also heterogeneous within each heart. When we plotted the maximum increase in anf mRNA expression (usually immediately subendocardial) versus heart weight to body weight ratio, there was a highly significant positive correlation between the level of anf mRNA expression and the overall magnitude of hypertrophic response (see Fig. 3). The changes in cftr mRNA expression were also variable. The mean epicardial to endocardial ratio of cftr mRNA expression in HF animals was 1.11 ± 0.02 (n = 5) which was statistically significantly lower than that in sham-operated animals (1.52 ± 0.06, see Fig. 3). The correlation between the ratio of epicardial to endocardial cftr mRNA expression and heart weight to body weight ratio just failed to reach statistical significance (P = 0.07, see Fig. 3).

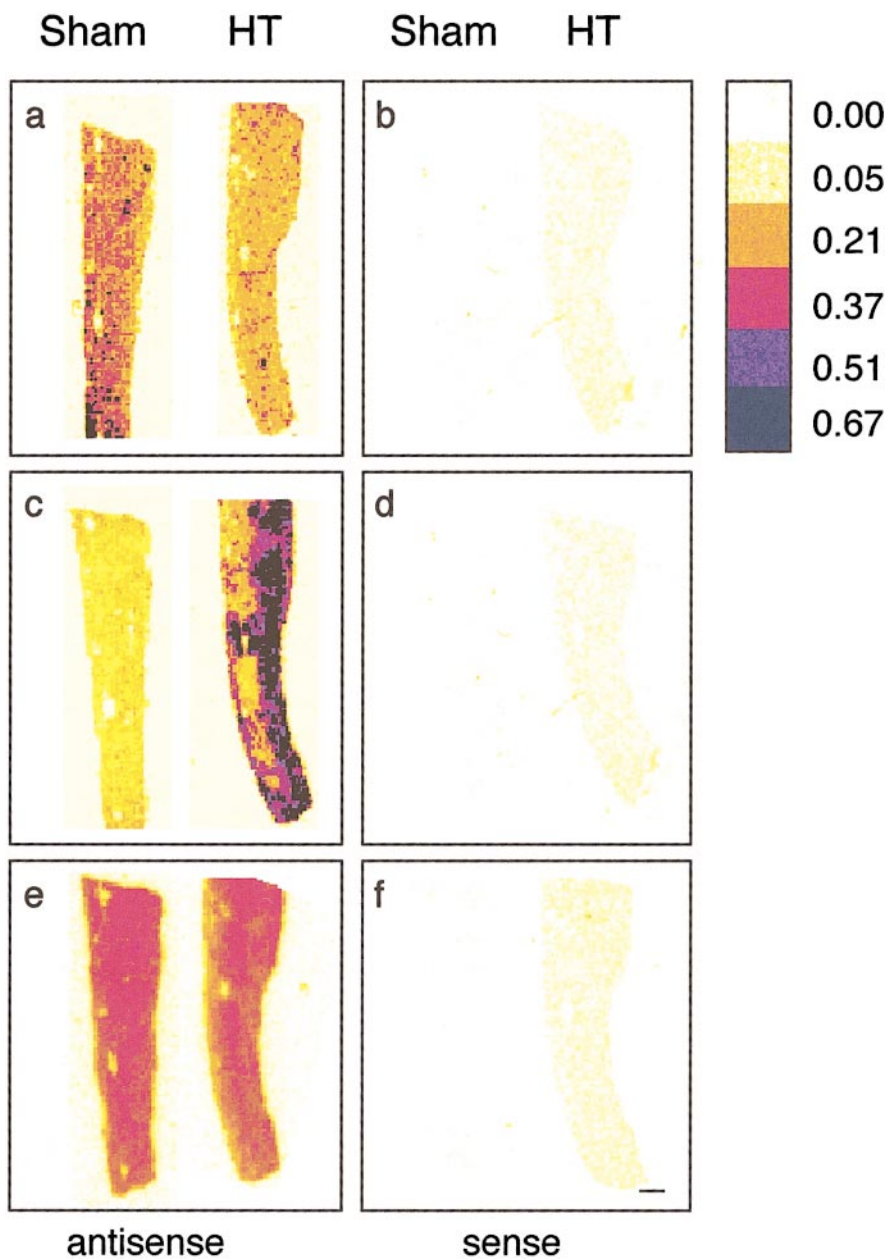

FIG. 1. *In situ* hybridization sections from sham-operated (sham) and heart failure (HF) rabbit hearts probed with antisense and sense riboprobes for (a, b) cftr, (c, d) anf and (e, f) gapdh. False color images were obtained using a linear transformation in NIH Image software (v. 1.62b7). The scale to the right indicates the densitometric values. The expression of cftr mRNA in the hearts from HF animals is lower and there is a loss of the normal epicardial to endocardial gradient of expression compared to the hearts of sham animals. Conversely, the expression of anf mRNA is increased in HF compared to sham animals and the increased expression is seen predominantly in the subendocardial regions. There are no significant differences in gapdh expression in HF compared to sham. Scale bars = 1 mm.

DISCUSSION

Following the induction of hypertrophy there was an overall increase in anf mRNA expression and decrease in cftr mRNA expression. The increase in anf mRNA was predominantly localised to subendocardial regions and the decrease in cftr mRNA expression was predominantly due to a decrease in expression in subepicardial layers (see Fig. 1). In both instances this represented a reversion to the embryonic pattern of gene expression (see Fig. 2 and [19, 24]), consistent with the hypothesis that heart failure is characterised by the reversion to an embryonic pattern of gene expression [14].

The increase in anf mRNA expression in subendocardial and perivascular regions is similar to that reported for spontaneous biventricular hypertrophy in

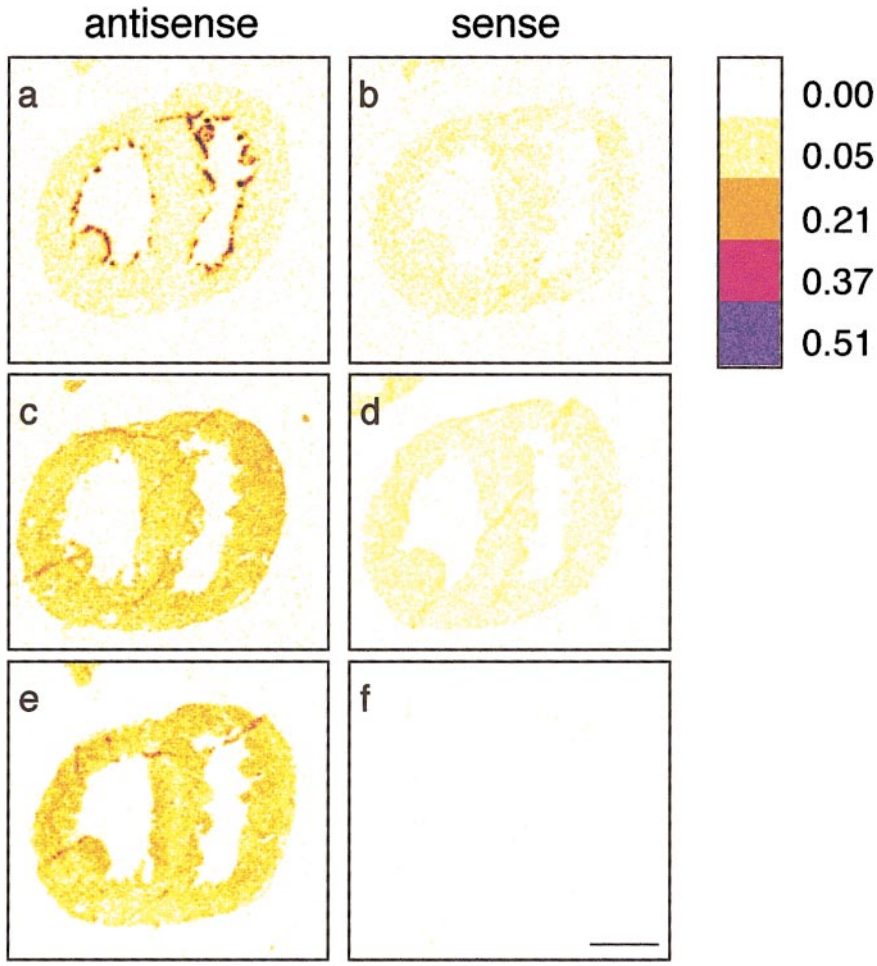

FIG. 2. *In situ* hybridization sections from embryonic day 30 (gestation = 31 days) rabbit hearts probed with antisense and sense riboprobes for (a, b) anf, (c, d) cftr, and (e, f) gapdh. False color images were obtained using a linear transformation in NIH Image software (v. 1.62b7). The scale to the right indicates the densitometric values. cftr and gapdh mRNA are expressed uniformly across the wall of the left ventricle whereas anf is expressed predominantly in the subendocardial regions. Scale bars = 1 mm.

the rat [15] and in patients with dilated cardiomyopathy [25]. The variable increase in anf mRNA expression we observed is also similar to that reported by Vikstrom *et al.* in a transgenic mouse model of hypertrophic cardiomyopathy [26].

The decrease in cftr mRNA expression was also quite variable (see Fig. 3). However, in the most severe cases of hypertrophy there was clearly a marked decrease in epicardial cftr mRNA expression. It is possible that changes in cftr mRNA expression may only occur during marked hypertrophy or relatively late in the hypertrophic response, hence the variability when measured two weeks after induction of pressure overload. An alternative explanation would be that the changes in mRNA expression during early stages of hypertrophy and/or milder insults are quite low and therefore would require the use of more sensitive techniques for detecting such changes.

The total changes in cftr mRNA expression that we observed in this study (1.5- to 2-fold decrease) are broadly similar to that observed for the decrease in cAMP-activated chloride channel activity in the pressure-overload model of hypertrophy in the guinea-pig [11]. This is consistent with our previous work indicating that there is a good correlation between the level of cftr mRNA expression and the density of cAMP-activated chloride channel activity in ventricular myocytes [23].

There are many previous studies that have reported decreases in repolarising ion current densities [6–11] as well as decreases in K^+ channel gene expression in various models of hypertrophy [9, 16–18]. This is the first report of decreased mRNA expression in cardiac hypertrophy. Furthermore, to our knowledge, this is the first report of reversion to both embryonic levels and embryonic patterns of distribution of any ion channel gene in cardiac hypertrophy. The normal rhythm of the heart beat is critically dependent on the normal pattern of spread of activation and repolarisation. In addition to the prolonged repolarisation caused by de-

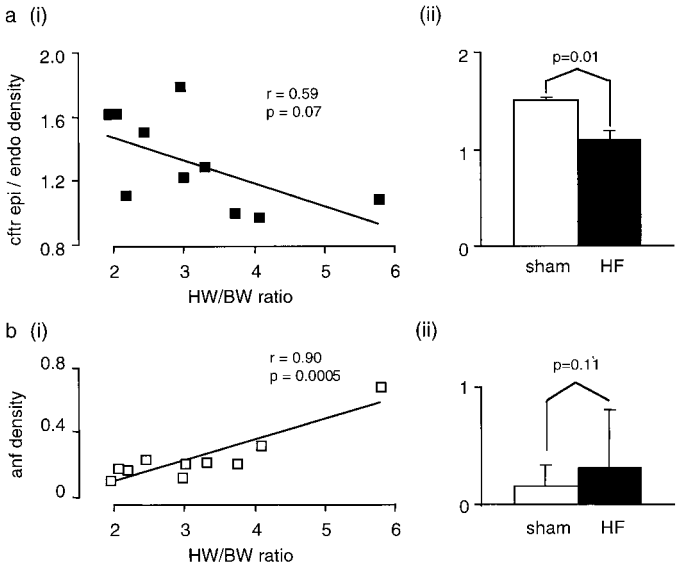

FIG. 3. (a) (i) The epicardial:endocardial ratio of cftr expression versus heart weight/body weight (HW/BW) ratio and (ii) the mean (±SEM) ratio of cftr expression in sham-operated (sham) versus heart failure (HF) animals. All values represent antisense–sense signals. The epicardial:endocardial ratio of cftr expression in HF animals was significantly lower than that in sham animals; however, the correlation between the epicardial:endocardial cftr ratio and HW/BW ratio just failed to reach statistical significance ($P = 0.07$). (b) (i) The maximum density of anf expression versus HW/BW ratio and (ii) the mean (±SEM) maximum anf expression in sham-operated versus HF animals. The correlation between maximum anf expression and HW/BW ratio was highly statistically significant ($P < 0.0005$); however, the mean value for maximum anf expression in HF animals was not significantly higher ($P = 0.11$) than that observed in sham animals.

creases in repolarising ion currents, our study suggests that changes in the patterns of repolarising ion channel gene expression could also be an important contributor to the increased risk of arrhythmias in hypertrophied and failing hearts.

Since Beuckelmann *et al.* [27] published the first report of a lengthening of the action potential in patients with terminal heart failure a number of different mechanisms have been suggested for this increased duration of the action potential [2]. A reduction in densities of K^+ currents is considered as a major determinant [5] but other mechanisms contribute. Although the precise role of CFTR in the human heart is still largely unknown [28, 29], it has recently been shown that a chloride current can be activated in response to β3-adrenergic stimulation [30] which would be consistent with a role for CFTR in the human heart. It has been suggested that the gradient of cftr may contribute to maintaining the normal gradient of repolarisation duration across the ventricular wall during adrenergic stimulation [31]. Thus decreased expression and loss of the gradient of cftr expression may contribute to an abnormal response to adrenergic stimulation and hence increase the risk of arrhythmias and sudden death in patients with heart failure.

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