Increased Expression of Isoform 1 of the Sarcoplasmic Reticulum Ca\(^{2+}\)-Release Channel in Failing Human Heart

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**Background**—The sarcoplasmic reticulum (SR) Ca\(^{2+}\)-release channel plays a key role in the excitation-contraction coupling of cardiac myocytes. Because respective alterations have been reported in human heart failure, we investigated isoform expression of the SR Ca\(^{2+}\)-release channel in human hearts from patients with terminal heart failure (dilated cardiomyopathy [DCM], n=8) and nonfailing organ donors (NF, n=8).

**Methods and Results**—Expression of mRNA of SR Ca\(^{2+}\)-release channel isoforms in isolated human cardiomyocytes and myocardial tissue was analyzed by reverse-transcription polymerase chain reaction. Protein expression was quantified in myocardial tissue with \[^{3}H\]-ryanodine binding and with Western blots, expressed as densitometric units per microgram of protein (DU), and cellular localization was visualized with immunohistochemistry. We found mRNA expression of isoforms 1, 2, and 3 in cardiomyocytes and myocardial tissue both in NF and DCM. Total SR Ca\(^{2+}\)-release channel protein expression in NF (B\(_{\text{max}}\) 2.16±0.43 pmol/mg protein) and in DCM (B\(_{\text{max}}\) 2.33±0.22 pmol/mg protein) myocardium was unchanged. Expression of isoform 1 of the SR Ca\(^{2+}\)-release channel was significantly (P=0.0037) increased in DCM myocardium (NF 1.97±0.25 versus DCM 3.37±0.31 DU), whereas protein expression of isoform 2 (NF 14.62±0.87 versus DCM 13.52±0.43 DU) and isoform 3 (NF 1.39±0.13 versus DCM 1.35±0.19 DU) was unchanged. All 3 isoforms of the protein could be localized in human ventricular myocytes with fluorescence immunohistochemistry.

**Conclusions**—All 3 isoforms of the SR Ca\(^{2+}\)-release channel were determined in human ventricular cardiomyocytes. Increased expression of isoform 1 of the SR Ca\(^{2+}\)-release channel could contribute to impaired excitation-contraction coupling in human heart failure. *(Circulation. 2001;103:2739-2744.)*

**Key Words:** sarcoplasmic reticulum | heart failure | calcium | receptors

Alterations in Ca\(^{2+}\) homeostasis\(^{1}\) and blunted \(\beta\)-adrenergic signal transduction\(^{2}\) appear to play a key role in the deterioration of cardiac contractile function in heart failure. The Ca\(^{2+}\) transient from failing human myocytes is reduced in amplitude and prolonged in time course.\(^{3}\) Therefore, both Ca\(^{2+}\) release from intracellular stores\(^{3}\) and reuptake into the sarcoplasmic reticulum (SR) appear to be impaired.\(^{4}\) In addition to changes in Ca\(^{2+}\) loading of the SR, altered function of the SR Ca\(^{2+}\)-release channel/ryanodine receptor is discussed as the cause of altered Ca\(^{2+}\) release in heart failure.\(^{5}\) However, SR Ca\(^{2+}\)-release channel expression in human heart failure appears to be unchanged, although the literature is inconclusive (for review, see Marks\(^{6}\)).

Nevertheless, changes in the function of a protein can also be related to different isoform expression, as shown, for example, for myosin heavy chain isoform expression in human heart failure.\(^{7}\) From the SR Ca\(^{2+}\)-release channel, 3 isoforms have been cloned. Isoform 1 of the SR Ca\(^{2+}\)-release channel is mainly expressed in skeletal muscle and appears to mediate voltage-induced Ca\(^{2+}\)-release and skeletal muscle-type excitation-contraction coupling. Isoform 2 of the SR Ca\(^{2+}\)-release channel is expressed primarily in the heart, and this isoform is attributed to Ca\(^{2+}\)-induced Ca\(^{2+}\) release and to cardiac-type excitation-contraction coupling. The third isoform is expressed in a wide variety of tissues at low levels, but its functional properties have not yet been resolved (for review, see Sutko et al\(^{8}\)). Recently, coexpression of the different isoforms has been reported for the mouse\(^{9}\) and porcine\(^{10}\) heart. However, isoform expression of the SR Ca\(^{2+}\)-release channel in the human heart and changes in isoform expression in the human heart during heart failure have not been determined. Therefore, we addressed the question of SR Ca\(^{2+}\)-release channel isoform expression in the human heart and quantified the changes in isoform expression in cardiac failure.

**Methods**

**Patients**

Left ventricular myocardium from nonfailing hearts from brain-dead organ donors (n=8, 5 men and 3 women aged 44±8 years) and from

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patients with terminal heart failure due to dilated cardiomyopathy (DCM; n=8, 6 men and 2 women aged 43±11 years; ejection fraction 28±5%) were investigated. Moreover, cardiac myocytes were isolated from the left ventricle of explanted hearts from 6 organ donors (4 men and 2 women aged 46±9 years) and 6 explanted hearts from patients with terminal heart failure due to DCM (3 men and 3 women aged 48±10 years). The preoperative clinical degree of heart failure in the DCM group was New York Heart Association class IV in all patients. Patients were taking regular medication, including vasodilators (nitrates and ACE inhibitors), cardiac glycosides, and diuretics. Patients gave written informed consent before the operation.

**Isolation of Ventricular Cardiomyocytes From Human Myocardium**

From 6 failing and 6 nonfailing hearts, a sample of the left ventricle (average weight 1 to 2 g) was digested with 4 U/mL type XXIV protease (Sigma) and 1 mg/mL collagenase type II (Biochrom) at 35°C for 30 minutes. To test specificity of myocyte isolation, we plated the cell suspension on culturing dishes and morphologically analyzed the cells under light microscopy (200× magnification). The vast majority of cells presented the typical rod-shaped morphology and striation of cardiomyocytes.

**Isolation of RNA From Human Cardiomyocytes**

RNA preparations from isolated cardiomyocytes or myocardial tissue were performed in accordance to the method of Chomczynski and Sacchi with further modifications as previously described for myocardial tissue and finally stored at −80°C until use for polymerase chain reaction (PCR) experiments. Concentration and purity of RNA were determined photometrically, and only RNA with a 260/280 ratio greater than 1.6 was used for further experiments.

**Reverse-Transcriptase PCR With RNA From Human Cardiomyocytes**

With 1 μg of total RNA, reverse transcription (RT) was performed with Expand Reverse Transcriptase (Roche) followed by amplification with specific primers for isoform 1 (forward primer TGA GGA ACC GCC TGA AGA AAA CCG G, reverse primer GAG GTG CAG CTC CTC CTC TGA), isoform 2 (forward primer AAT CCA GAA CGG GCC GAG ATG, reverse primer GTA ATG TCT TCG ACC CAC ACG), and isoform 3 (forward primer CAC TCC TCC TTC AGC CAC AGC, reverse primer ACT CGT CCG AGA AGA GCC AGA) (MWG Biotech) for the SR Ca\(^{2+}\)-release channel isoforms with 50 U of Expand High Fidelity PCR System (Roche) according to the vendor’s protocol. DNA was separated in 0.6% agarose (Merck) horizontal gel containing 0.1% ethidium bromide (Sigma), and gels were viewed and photographed in a Bio-Rad gel documenter (Bio-Rad Laboratories).

**Tissue Preparation for Protein Preparation**

Myocardium (0.5 to 1 g) from the free left ventricular wall was powdered in liquid nitrogen, and homogenates were prepared as previously described. Homogenates were suspended in buffer (in mmol/L: KCl 0.4, HEPES 40, Tris 20, EDTA 10, Na\(_2\)PO\(_4\) 50, pH 7.2) (Merck) and stored at −80°C until use in Western blot experiments. After homogenization, crude membranes (U3) were purified for \(^{3}H\)-ryanodine binding assay, also as described previously.

**\(^{3}H\)-Ryanodine Binding**

Protein (50 μg) U3 was incubated in 500 μL of incubation buffer (KCl 1 mol/L [Merck], phenylmethylsulfonylfluoride 5 μmol/L, piperazine-N,N'-bis[2-ethanesulfonic acid] 25 mmol/L, CaCl\(_2\), 500 μmol/L [Bio-Rad], ATP 3 mmol/L [Sigma], pH 7.4) for 90 minutes at 37°C with increasing concentrations of \(^{3}H\)-ryanodine (Amersham, Pharmacia Biotech) from 0.1 to 40 nmol/L and counted in a Beckman β-counter (Beckman Instruments). The 8-point binding kinetics were calculated by linear curve fitting with GraphPad Prism software.

**Analysis for Protein Isoform Expression**

Immunoblots in myocardial homogenates were performed according to Towbin et al with modifications as described previously. The antibody against isoform 1 of the ryanodine receptor was a sheep polyclonal IgG antibody directed against rabbit skeletal muscle ryanodine receptor from Upstate Biotechnology (No. 06-410); mouse monoclonal IgM antibody against type 1 rabbit SR Ca\(^{2+}\)-release channel was also from Upstate Biotechnology (No. 05-269). The antibody against isoform 2 of the ryanodine receptor was a mouse monoclonal IgG antibody against the canine isoform 2 SR Ca\(^{2+}\)-release channel purchased from ABR (Affinity Bioreagents Inc; No. MA3-916), and the antibody against isoform 3 of the ryanodine receptor was a goat polyclonal IgG antibody against rabbit SR Ca\(^{2+}\)-release channel from Upstate Biotechnology (No. 06-416).

The secondary peroxidase-conjugated antibodies were monoclonal sheep anti-mouse IgG antibodies for use with monoclonal type 1 and type 2 antibodies and goat IgG antibodies used for polyclonal type 1 and type 3 antibodies (Sigma Immunochemicals). Linearity of the protein application to the densitometric signal was determined from 25 to 250 μg of protein. The correlation of protein to densitometric signal was calculated by a linear regression curve fit (GraphPad Prism). The correlation between protein application and the densitometric signal was r=0.88 for isoform 1 of the SR Ca\(^{2+}\)-release channel, r=0.93 for isoform 2, and r=0.96 for isoform 3. For quantitative Western blots, 100 μg of protein was used per slot, which was in the linear range for protein application. Quantification of protein expression was achieved after the films were scanned into a personal computer. Care was taken to place the regions of interest over the respective band at 100 to 300 kDa and the second band below 200 kDa. Analysis of densitometric volume of bands was made with a commercially available computer program (ImageQuant).

**Immunohistochemistry in Human Myocardium**

Cryosections from human left ventricular myocardium from failing and nonfailing hearts were prepared as previously described with further modifications. Slices were incubated with the same primary antibodies against SR Ca\(^{2+}\)-release channel isoforms as used for Western blots. The secondary antibody was a biotinylated goat anti-rabbit or anti-mouse antibody (Dako). Finally, slices were labeled with streptavidin-conjugated peroxidase (Amersham, Pharmacia). Slices were stained with 3,3′-diaminobenzidine as the chromogen and viewed and photographed under a Zeiss Axiovert microscope (Carl Zeiss).

**Statistical Analysis**

The means of n=8 DCM and n=8 nonfailing hearts are given with SEM values. Densitometric units (DU) were normalized to protein concentration applied per slot and expressed as densitometric units per microgram of protein. Differences between groups were compared with a 2-way ANOVA.

**Results**

**RNA Expression of Isoforms 1, 2, and 3 of the SR Ca\(^{2+}\)-Release Channel**

RNA expression of the skeletal isoform 1, the cardiac isoform 2, and the ubiquitous isoform 3 of the SR Ca\(^{2+}\)-release channel was detected in left ventricular myocardial tissue from both failing and DCM patients (Figure 1). Additionally, RNA expression of these 3 isoforms was detected specifically in cardiomyocytes isolated from the left ventricle of explanted hearts from failing and DCM patients (Figure 2).
The \([^{3}H]\)-ryanodine binding experiments in myocardial homogenates from nonfailing and terminally failing patients revealed comparable \((P\leq0.38)\) affinity of \([^{3}H]\)-ryanodine to the SR Ca\(^{2+}\)-release channel, indicated in the \(K_{d}\) value of 0.25±0.02 nmol for nonfailing and 0.29±0.22 nmol for failing myocardium. The absolute amount of \([^{3}H]\)-ryanodine binding sites was also comparable \((P\leq0.10)\) in nonfailing \((B_{max} 2.16\pm0.43 \text{ pmol/mg protein})\) and terminally failing \((B_{max} 2.33\pm0.22 \text{ pmol/mg protein})\) myocardium (Figure 3).

**Protein Expression of Isoforms 1, 2, and 3 of the SR Ca\(^{2+}\)-Release Channel**

Overall protein expression with Coomassie blue staining demonstrated an intensive band between 200 and 300 kDa corresponding to the specific detection of SR Ca\(^{2+}\)-release channel isoforms with immunoblots (Figure 4). Skeletal isoform 1 could be detected between 200 and 300 kDa in nonfailing and DCM human hearts both with the polyclonal (see Figure 5) and the monoclonal antibody against type 1 of the SR Ca\(^{2+}\)-release channel. Expression of isoform 1 was significantly higher \((P\leq0.0037)\) in the left ventricle of DCM hearts \((3.37\pm0.31 \text{ DU/\mu g tissue})\) than in nonfailing controls \((1.97\pm0.25 \text{ DU/\mu g tissue})\) (Figure 5).

Cardiac isoform 2 (Figure 6) and isoform 3 of the SR Ca\(^{2+}\)-release channel were also detected in the left ventricle from nonfailing and DCM hearts (Figure 7). The expression of isoform 2 (nonfailing 14.62±0.87; DCM 13.52±0.43 DU/\mu g tissue) and isoform 3 (nonfailing 1.39±0.13; DCM 1.35±0.19 DU/\mu g tissue) was unchanged during heart failure. Calsequestrin expression as an internal standard for protein expression was unchanged in the left ventricle from nonfailing and failing hearts (nonfailing 83.9±6.2; DCM 81.7±7.0 DU/\mu g protein).
Cellular Localization of SR Ca\(^{2+}\)-Release Channel Isoforms

Cellular localization of isoforms 1, 2, and 3 of the SR Ca\(^{2+}\)-release channel in human cardiac myocytes could be determined in human myocardial tissue. Figure 8 shows histological details of distribution of the SR Ca\(^{2+}\)-release channel for isoforms 1, 2, and 3 in cryosections of human myocardium.

Discussion

In the present report, we provide evidence for the expression of all 3 isoforms of the SR Ca\(^{2+}\)-release channel in the human heart. During heart failure, specific changes in isoform expression were found with increased expression of isoform 1 of the SR Ca\(^{2+}\)-release channel in human failing cardiomyocytes. Coexpression of the different isoforms of the SR Ca\(^{2+}\)-release channel in human cardiomyocytes is a novel finding; however, different investigators have described this phenomenon in various animal species and different organs, particularly in the brain. In the heart, coexpression of the SR Ca\(^{2+}\)-release channel isoform 1 and isoform 2 by PCR methods was reported in mouse cardiac tissue, and expression of isoforms 2 and 3 has been determined in porcine cardiac tissue. Moreover, in our laboratory, all 3 isoforms of the SR Ca\(^{2+}\)-release channel were discovered on the mRNA and protein levels in different myocardial chambers of the nonfailing heart.

The detection of isoform 1 in the human heart is surprising, but cross-reaction with the other isoforms is highly unlikely. For detection of isoform 1 of the ryanodine receptor, we performed Western blotting and immunohistochemistry with 2 different antibodies against the respective ryanodine channel isoform. McPherson and Campbell showed high isoform selectivity for both the monoclonal antibody against type 1 (entitled XA7 in their report) and the polyclonal antibody against type 1 (entitled GP-23). The antibody against the type 3 SR Ca\(^{2+}\)-release channel that we used was also tested for isoform specificity by McPherson and Campbell and was entitled goat-43 in their study. Moreover, in a previous study, we performed experiments on the specificity of the antibodies by analysis of the expression in different human organs, such as striated muscle, cortex, cerebellum, and heart. The expression of all 3 of the SR Ca\(^{2+}\)-release channel isoforms could be shown by RT-PCR experiments with mRNA prepared from isolated cardiomyocytes of the left ventricle. Moreover, cellular localization was determined with immunohistochemistry in human myocardial tissue sec-

Figure 5. Protein expression of isoform 1 of SR Ca\(^{2+}\)-release channel in left ventricular myocardium from explanted human hearts. Myocardium from 8 explanted human hearts from nonfailing organ donors (NF) and from patients with terminal heart failure due to DCM were investigated. Top, Representative Western blots with specific band between 200 and 300 kDa. Bottom, Quantitative analysis of mean±SEM of 8 experiments. Significance of different protein expression is indicated by asterisk.

Figure 6. Protein expression of isoform 2 of SR Ca\(^{2+}\)-release channel in left ventricular myocardium from explanted human hearts. Myocardium from 8 explanted human hearts from nonfailing organ donors (NF) and from patients with terminal heart failure due to DCM were investigated. Top, Representative Western blots with specific band between 200 and 300 kDa. Bottom, Quantitative analysis of mean±SEM of 8 experiments.

Figure 7. Protein expression of isoform 3 of SR Ca\(^{2+}\)-release channel in left ventricular myocardium from explanted human hearts. Myocardium from 8 explanted human hearts from nonfailing organ donors (NF) and from patients with terminal heart failure due to DCM were investigated. Top, Representative Western blots with specific band between 200 and 300 kDa. Bottom, Quantitative analysis of mean±SEM of 8 experiments.
ions. Thus, determination of the isoforms in other cells (i.e., fibroblasts) contaminating the preparations can be excluded, and the expression of all 3 isoforms of the SR Ca\(^{2+}\)-release channel can be specifically localized to cardiomyocytes of the left ventricle of human heart. This receptor equipment of the myocyte might allow a finer and more complex regulation of excitation-contraction coupling in the human heart.

In human heart failure and in different animal models of hypertrophy and failure, altered excitation-contraction coupling has been described. The underlying mechanism, however, has not yet been resolved. Data from Ca\(^{2+}\) spark experiments, in which SR Ca\(^{2+}\)-release is visualized by the fluorescent dye fluo-3, suggest a defect in Ca\(^{2+}\)-induced Ca\(^{2+}\)-release in failing and hypertrophied rat hearts. However, despite unchanged overall protein expression of the SR Ca\(^{2+}\)-release channel in failing myocardium, a shift of isoform expression in failing human myocardium with an increased expression of the isoform 1 was discovered in the present study. The shift of isoform expression of the SR Ca\(^{2+}\)-release channel is in agreement with the isoform shifts in cardiac failure previously described for contractile proteins such as myosin\(^{7}\) and actin, as well as for Ca\(^{2+}\)-regulating proteins such as Na\(^+/K\(^{-}\))-ATPase. Because the isoforms are attributed to different Ca\(^{2+}\) release properties, an increase in isoform 1 of the SR Ca\(^{2+}\)-release channel would result in functional changes of excitation-contraction coupling during heart failure, allowing voltage-induced Ca\(^{2+}\) release in the failing heart. In addition to the Ca\(^{2+}\)-induced Ca\(^{2+}\) release mechanism, the failing myocardium would have an alternative Ca\(^{2+}\) release mechanism by voltage-gated Ca\(^{2+}\) release. Functional studies for both Ca\(^{2+}\)-induced Ca\(^{2+}\) release and voltage-induced Ca\(^{2+}\) release are available for cardiac muscle. However, voltage-induced Ca\(^{2+}\) release in the heart is discussed very controversially and has been questioned by other investigators. Thus, it remains to be established whether voltage-induced Ca\(^{2+}\) release is also a mechanism for excitation-contraction coupling in the human heart and is particularly present in failing human myocytes. Therefore, the functional relevance of this isoform shift of protein expression of SR Ca\(^{2+}\)-release channel is a subject for further investigations.

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