Inhibitory Cardiac Transcription Factor, SRF-N, Is Generated by Caspase 3 Cleavage in Human Heart Failure and Attenuated by Ventricular Unloading

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Background—Knowledge about molecular mechanisms leading to heart failure is still limited, but reduced gene activities and modest activation of caspase 3 are hallmarks of end-stage heart failure. We postulated that serum response factor (SRF), a central cardiac transcription factor, might be a cleavage target for modest activated caspase 3, and this cleavage of SRF may play a dominant inhibitory role in propelling hearts toward failure.

Methods and Results—We examined SRF protein levels from cardiac samples taken at the time of transplantation in 13 patients with end-stage heart failure and 7 normal hearts. Full-length SRF was markedly reduced and processed into 55- and 32-kDa subfragments in all failing hearts. SRF was intact in normal samples. In contrast, the hearts of 10 patients with left ventricular assist devices showed minimal SRF fragmentation. Specific antibodies to N- and C-terminal SRF sequences and site-directed mutagenesis revealed 2 alternative caspase 3 cleavage sites, so that 2 fragments were detected of each containing either the N- or C-terminal SRF. Expression of SRF-N, the 32-kDa fragment, in myogenic cells inhibited the transcriptional activity of α-actin gene promoters by 50% to 60%, which suggests that truncated SRF functioned as a dominant-negative transcription factor.

Conclusions—Caspase 3 activation in heart failure sequentially cleaved SRF and generated a dominant-negative transcription factor, which may explain the depression of cardiac-specific genes. Moreover, caspase 3 activation may be reversible in the failing heart with ventricular unloading. (Circulation. 2003;108:407-413.)

Key Words: heart failure ■ serum response factor ■ apoptosis ■ heart-assist device
sible for the suppression of cardiac-specific gene transcription and (2) caspase 3 activation may be reversible with ventricular unloading.

Methods

Human Failing Heart Tissues

Myocardial samples were obtained from 13 patients with end-stage heart failure at the time of transplantation: 7 patients had ischemic cardiomyopathy, 5 had dilated cardiomyopathy, and 1 had hypertrophic cardiomyopathy. An additional 10 patients, including 8 with dilated cardiomyopathy and 2 with ischemic cardiomyopathy, had been maintained on LVAD until transplantation. Samples were obtained from 7 patients who died of noncardiac causes as a control group. Left ventricular ejection fraction was <20% in all heart failure patients. The Institutional Review Board of Baylor College of Medicine approved all protocols.

Plasmid Constructs and Syntheses of Recombinant SRFs

The construction and use of reporter plasmids containing the skeletal (SK-luc) and cardiac α-actin (cCA-luc) promoters linked to luciferase reporters were as described previously.19,20 pCGN, a cytomegalovirus promoter-driven expression vector, was used to express Nkx2.5, GATA4, full-length SRF (pCGN-SRF), SRF-N (residues 1 to 254; pCGN-SRF-N), SRF-C (residues 255 to 508; pCGN-SRF-C), and SRFpmy1 (pCGN-SRFpmy1) containing triple point mutants within the MADS box.21

SRF and SRF-N expression plasmids were constructed by transferring SRF coding sequences from the respective pCGN-SRF plasmids as XhoI-EcoRI fragments. To make individual mutated recombinant SRFs, we substituted alanine (A) for glutamic acid (E) and aspartate (D) at E243 and D245 as A245 or E252 and D254 as A254. The combination of A245 and A254 made A245/A254. The QuickChange site-directed mutagenesis kit (Stratagene) was used with the primers 5'-CCACCTGGCTTTGGAGCGACAGGCTCTCACCCAGGG, 5'-CTACACCGGGTGCCGCTCTGCGACACACTGAAGC. Proteins were expressed in BL21 (Stratagene) competent cells, followed by nickel-nitrilotriacetic acid resin purification.

Cell Cultures, Plasmid DNA Transfection, and Reporter Gene Assays

CV1 fibroblasts, C2C12 myoblasts, and neonatal cardiomyocytes were cultured as described previously.19,20 Cells plated in 30-mm plates were transfected with 0.6 μg of total plasmid DNA containing the reporter plasmids and the indicated expression plasmids. Transfections were performed with LipofectAMINE (Invitrogen). Cells were harvested 40 hours after transfection, and luciferase activity was measured as described previously.19

Western Blot Analysis

An anti-SRF N-terminal antibody (anti-SRF-N) was generated (Bethyl Laboratories) with the peptide sequence GANGGRVPGNGA, a portion of exon 1 domain of SRF from human origin. An anti-SRF C-terminal antibody (anti-SRF-C) was purchased (Santa Cruz). These antibodies were tested against SRF-N, SRF-C, and full-length SRF expressed in transfected CV1 cells. An anti-caspase 3 antibody was purchased (Santa Cruz). Protein samples were prepared and separated as described previously.22 Even loadings were confirmed by Ponceau staining.

Immunofluorescence Analysis

Visualization of transfected SRF and SRF-N cellular localization was implemented in neonatal rat cardiomyocytes attached to coverslips as described previously.23

Electrophoretic Gel Mobility Shift Assay

DNA binding activity was assayed by electrophoretic gel mobility shift assay (EMSA) with recombinant SRF-N and full-length SRF and with the oligonucleotide probe corresponding to the proximal SRE1 (serum response element) of the skeletal α-actin promoter, as described previously.19,20

In Vitro Cleavage of SRF by Caspase 3

Recombinant SRF was incubated for 2, 4, and 6 hours with active caspase 3 (BD PharMingen; SRF:caspase 3, 50:1 [wt/wt]) in the presence or absence of 20 μmol/L Z-VDAD-fmk (BD PharMingen), a caspase 3 inhibitor, in a reaction buffer (50 mmol/L HEPES [pH 7.4], 100 mmol/L NaCl, 1 mmol/L EDTA, 0.1% CHAPS, 10 mmol/L DTT, and 10% glycerol).

Statistical Analysis

Data were analyzed by 1-way ANOVA followed by Bonferroni test. For those with equal variance test failure, Kruskal-Wallis test on ranks was used followed by Dunn’s method (SigmaStat, SPSS Inc). A value of P<0.05 was considered significant. Data are presented as mean±SEM.

Results

SRF Is Cleaved by Caspase 3, and Cleavage Is Attenuated by LVAD

We observed marked reduction of SRF accompanied by the appearance of 2 smaller fragments with molecular weights of 55 and 32 kDa in failing hearts (Figure 1A). Normal hearts barely contained cleavage products. These data were compared with 5 representative samples taken from the hearts of patients with equivalent heart failure who had been placed on an LVAD. The amount of SRF cleavage was strikingly reduced, and a substantial portion of full-length SRF was restored (Figures 1A and 1B). In fact, SRF cleavage in the LVAD group did not differ significantly from control (Figure 1B). On the basis of previous studies with apoptosis,17,18 we postulated that SRF cleavage was effected by caspase 3.

We also observed that these failing hearts had increased activated caspase 3 levels compared with normal hearts and failing hearts with LVAD (Figure 1C). The precursor level of caspase 3 was not significantly different among the 3 groups. Thus, the data suggest a correlation between increased activated caspase 3 levels in failing hearts and the proteolytic processing of full-length SRF with the concomitant appearance of SRF subspecies.

In Vitro Cleavage of SRF With Activated Caspase 3 and Comparison to Human Hearts

To confirm the role of caspase 3 in SRF cleavage, we incubated recombinant SRF with active caspase 3 (Figure 2B). This resulted in cleavage to native SRF with the generation of a 32-kDa fragment. The cleavage continued over 6 hours while the 32-kDa fragment gradually increased. The cleavage was completely inhibited by Z-VDAD. With the comparison of human heart, recombinant SRF cleaved by active caspase 3 was run with human heart tissues on the same gel, which showed that 32-kDa subspecies comigrated (Figure 2C). However, the 55-kDa fragment was not observed (Figures 2B and 2C) in vitro, which suggests that only the 32-kDa SRF subspecies is associated with caspase 3 in human heart. Other proteases, including calpain, might be involved in generating the 55-kDa fragment. Calpain is considered responsible for cardiac troponin I cleavage under pathophysiological conditions.24
Caspase 3 Cleavage of SRF Is Defined by Mutation of SRF and N-Terminal and C-Terminal Specific Antibodies

On the basis of the observation from human hearts and the in vitro cleavage, caspase 3 cleavage appeared to have occurred near the center of SRF. Examination of SRF protein sequences revealed 2 possible caspase consensus cut sites at the 245th and 254th aspartate residues. Cleavage at either site would generate 2 32-kDa fragments, as shown in Figure 2A. To precisely map the clipping sites, mutated SRF species A245, A254, and A245/A254 (Figure 2A) were treated with caspase 3, and only the A245/A254 mutant showed a complete block to caspase 3 (Figure 2C), which indicates that both native sites are subject to caspase 3 cutting. To extend this observation to failing human hearts, we generated specific antibodies against the N- and C-terminal ends of SRF. The specificity of anti-SRF-N and -C antibody was evaluated by Western blot analyses of SRF-N, SRF-C, and full-length SRF expressed in transfected CV1 cells. As shown in Figure 3A, anti-SRF-N and anti-SRF-C detected SRF-N and SRF-C fragments, respectively, as well as full-length SRF, which indicates specific recognition. We observed that the 32-kDa fragment found in heart samples reacted with both antibodies (Figure 3B). The same result was observed for in vitro recombinant SRF cleavage experiment (data not shown). Thus, the cleavage by caspase 3 occurs at D245 and D254, which generates 2 different 32-kDa fragments, SRF-N and SRF-C, in failing hearts.

SRF-N Serves as a Dominant-Negative Inhibitor for Muscle-Specific Genes

Because SRF-N contains an intact MADS box without an activation domain, we hypothesized that it bound SRE DNA and functioned as a dominant-negative transcription factor. We asked whether SRF-N translocated to the nucleus. Figure 4A shows immunofluorescence of SRF wild type and SRF-N in the nuclei of transfected cardiac myocytes, which indicates unaltered capability of SRF-N for nuclei localization. With regard to SRF-N DNA binding activity, EMSA was performed, as shown in Figure 4B. The SRF-N fragment bound to the SRE1 DNA probe as well as the full-length SRF, which suggests that SRF-N retained DNA binding activity and could compete with the endogenous SRF.

To evaluate potential functional significance of the cleaved fragments, we overexpressed SRF-N and SRF-C via cytomegalovirus promoter-driven expression plasmids cotransfected with cardiac α-actin and skeletal α-actin promoter reporter plasmids. As shown in Figures 5A and 5B, SRF-N significantly decreased the basal transcriptional activities of cardiac α-actin and skeletal α-actin promoter by 60% and 50% in cardiomyocytes and C2C12 myoblasts, respectively, which suggests that this fragment functioned as a dominant-negative inhibitor of SRF-dependent transcription. This inhibitory effect was similar to that observed by an SRF-negative mutant, SRFpml.20,21,25 In contrast, the 32-kDa fragment from the C terminal, SRF-C, did not inhibit actin promoter activity in both cells and actually caused a modest
Figure 2. A. Schematic presentation of 2 cleavage sites, aspartate 245 and 254, and their putative SRF fragments, 32 kDa, derived from cleavage by activated caspase 3. Mutation sites for each mutated SRF species are indicated. B, Representative Western blot showed that recombinant SRF was cleaved by active caspase 3 in vitro, and cleavage yielded 32-kDa fragment. Cleavage was blocked by caspase 3 inhibitor, Z-VAD. (+) indicates recombinant SRF as positive control. C, 32-kDa fragment derived from recombinant SRF cleavage by activated caspase 3 in vitro corresponded in size to one detected in failing human hearts by Western blot. Mutated SRF species A_{245}, A_{254}, and A_{245/A_{254}} were treated with caspase 3 and showed complete block to caspase 3 only in A_{245/A_{254}} mutant.
increase versus the control. To control for endogenous cardiogenic factors and high levels of SRF, we cotransfected 2 SRF cofactors, Nkx2.5 and GATA4, with SRF and its 2 subspecies at different combinations in CV1 fibroblasts. As indicated in Figures 5C and 5D, SRF alone and a combination of SRF, Nkx2.5, and GATA4 both increased cardiac actin transactivity. SRF-N significantly blocked transactivation by 50% and 40%, respectively, consistent with the previous observation. SRF-C, however, failed to change transcription activity, which indicates that SRF-C may have a neutral role.

Discussion

SRF cleavage occurred in the myocytes of severely failing hearts. In noncardiac cell culture, cleavage of SRF was associated with apoptosis being mediated by caspase,17,18 and the expression of a noncleavable SRF significantly suppressed apoptosis.17 Moretti and coworkers16 have demonstrated caspase 3–mediated cleavage of essential myosin light chain in failing myocardium and suggested that cleavage of cardiac-specific proteins may be part of the course of severe heart failure.

Figure 3. Caspase 3-dependent cleavage of SRF is documented by specific N- and C-terminal immunoreactivity. A, SRF-N, SRF-C, and full-length SRF were expressed in transfected CV1 cells. Cell lysates were analyzed by Western blot; SRF-N and SRF-C were detected exclusively by anti-SRF-N and anti-SRF-C antibody, respectively. However, full-length SRF was recognized by both antibodies, which indicates specificities of antibodies (NS indicates nonspecific). B, SRF fragments were recognized by both antibodies, which suggests combination of both N- and C-terminal domain fragments as schematized in Figure 2A. HR indicates heart.

Figure 4. A, Immunofluorescence analysis for SRF-N and SRF showing expression and capability of dominant-negative SRF-N to localize to myogenic nuclei, as well as SRF in transfected cardiac myocytes. B, Binding of SRF-N to SRE was demonstrated by EMSA.
heart failure. In the present study, we extended these results by examining (1) the application to human heart failure, (2) the potential reversibility of the caspase-induced proteolytic cascade, and (3) the amplified impact of SRF cleavage by virtue of the loss of a critical transcriptional modifier and the generation of its dominant-negative modifier.

Because these data are from human hearts, the concept of reversibility can only be inferred. The results demonstrated that the fragmentation in the LVAD group was not significantly different from a control group. There is no question that the LVAD did not cure the reason for failing myocardium; it merely unloaded the ventricle. Thus, one might postulate that caspase activation could be induced as a result of myocardial mechanical overload and that the reduction of this load would result in a clearing of activated caspase 3 and synthesis of new SRF.

Severe heart failure is associated with reduction of many cardiac-specific genes. However, which proteins would be chosen for proteolysis and the specificity of those choices has not been studied extensively. In the present report, we suggest another possibility in which specific cleavage of a transcription factor and generation of its dominant-negative modifier results in a more amplified effect on cardiac-specific genes. Without the transactivation domain, so that it competes with full-length intact MADS box but does not contain the C-terminal transactivation domain, SRF-N is generated and functions as a dominant-negative modifier. This fragment contains an alternatively spliced mutant SRF, which functions as a dominant-negative inhibitor in vivo by competing with native SRF for DNA binding in a manner similar to the SRF-N fragment. Recently, Davis and colleagues described small SRF species ascribed as alternatively spliced SRF transcripts in the failing human heart. We made a concerted effort at the level of RNase protection and cDNA cloning and were unsuccessful in determining alternative splicing of SRF transcript in the end-stage failing heart. Although alternative splicing of SRF may play an earlier role, it is our opinion that alternative splicing is not the key mechanism in human end-stage heart failure.

Thus, in the present study, it appears that there are 2 mechanisms involved in the suppression of SRF-dependent gene activation. First, there is a striking reduction in naturally occurring SRF. In addition, SRF-N is generated and functions as a dominant-negative modifier. This fragment contains an intact MADS box but does not contain the C-terminal transactivation domain, so that it competes with full-length SRF for binding to SRE. Without the transactivation domain, SRF-N binds but does not transactivate. A transgenic mouse model containing a mutated SRF has recently been reported to develop a severely dilated cardiomyopathy; we speculate that the pathological process is similar to human studies we present here, in which it is naturally occurring.

In summary, the results suggest that SRF is one of the targets for activated caspase 3 in failing cardiac myocytes in the absence of large-scale apoptosis. Cleavage results in reduction of native SRF and the generation of a dominant-negative inhibitor. Both effects may explain in part the marked reduction in cardiac-specific proteins associated with severe heart failure. Another striking feature of the present studies is the apparent reversibility of this process by ventricular unloading. The therapeutic significance remains spe-
ulative; certainly, long-term caspase inhibition has many hazards. It might be suggested, however, that intermittent short-term treatment with caspase inhibitors could have significant beneficial effects on the expression of cardiogenic-specific genes, many of which have relatively long half-lives.

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