

TRANSCRIPTIONAL REGULATION OF CARDIAC SARCOPLASMIC RETICULUM CALCIUM-ATPase GENE DURING THE PROGRESSION OF SEPSIS

Guang Wu,* Shaw-Lang Yang,† Chin Hsu,† Rei-Cheng Yang,† Hseng-Kuang Hsu,† Naikui Liu,* Jun Yang,* Lin-Wang Dong,* and Maw-Shung Liu*

*Department of Pharmacological and Physiological Science, St. Louis University School of Medicine, St. Louis, Missouri 63104; and †Department of Physiology, Kaohsiung Medical University, Kaohsiung, Taiwan

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ABSTRACT—Changes in sarcoplasmic reticulum Ca²⁺-ATPase (SERCA2a) gene expression in the rat heart during different phases of sepsis were studied. Sepsis was induced by cecal ligation and puncture (CLP). Septic rats were divided into two groups: the early hyperdynamic (9 h after CLP, early sepsis) and the late hypodynamic (18 h after CLP; late sepsis) groups. Western blot analyses reveal that SERCA2a protein level remained unaltered during early sepsis but was decreased by 59% during late sepsis. Northern blot analyses show that the steady-state level of SERCA2a mRNA stayed unchanged during the early phase but was decreased by 43% during the late phase of sepsis. Nuclear runoff assays show that the transcription rate of SERCA2a gene transcript remained unaffected during early sepsis but was decreased by 34% during late sepsis. The actinomycin D pulse-chase studies indicate that the half-life of SERCA2a mRNA was unaffected during the early and the late phases of sepsis. These findings demonstrate that during the early phase of sepsis, the protein level, the mRNA abundance, and the transcription rate of SERCA2a remained unaltered, whereas during the late phase of sepsis, the rate of transcription of SERCA2a was decreased, and the decreased transcription rate was associated with decreases in SERCA2a mRNA abundance and SERCA2a protein level in the rat heart. Based on these data, it is concluded that SERCA2a gene expression decreased during the late phase of sepsis in the rat heart and that the decreased expression was regulated at the transcriptional level.

KEYWORDS—Ca²⁺-transporting ATPase, Ca²⁺ uptake, Ca²⁺ homeostasis, cardiac dysfunction, gene expression, rat heart myocardium, septic shock

INTRODUCTION

Altered cardiac function is one of the key unresolved issues in the development of multiple organ system failure during the progression of sepsis (1, 2). In an animal model in which sepsis is induced by cecal ligation and puncture (CLP), the altered cardiac function is characterized by an initial hyperdynamic (elevated cardiac output, heart rate, and +LV dP/dt_{max}) followed by a late hypodynamic (diminished cardiac output, heart rate, mean arterial blood pressure, +LV dP/dt_{max}, and -dP/dt_{max}) responses (3). Sarcoplasmic reticulum (SR) Ca²⁺-ATPase (SERCA2a), in combination with other SR proteins such as phospholamban (PLB) and ryanodine-sensitive calcium release channel, play an important role in the regulation of cytosolic Ca²⁺ concentration, thereby modulating myocardial contractility (4, 5). Under physiological conditions, Ca²⁺ stored in the SR is released into cytosolic compartment through the opening of the ryanodine-sensitive calcium release channel and thereby interacts with contractile proteins to activate contraction. Subsequent reaccumulation of Ca²⁺ into the

SR through SERCA2a removes the bound Ca²⁺ from contractile proteins and thus initiates relaxation (4–7). Under pathological conditions, SERCA2a has been implicated in playing a critical role in the pathogenesis of cardiac dysfunction (8–12). The SERCA2a mRNA levels were reported to be decreased in hamsters with hereditary cardiomyopathy (8), in rats with myocardial infarction (9), in guinea pigs with chronic heart failure (10), and in humans with heart failure (6, 7, 11). The SERCA2a enzyme activity and protein content were found to be reduced in human heart failure, and the reductions were correlated with a depression in cardiac function (11). In pressure- and volume-overload cardiac hypertrophy, the SERCA2a gene expression was reduced, and the reduction in the SERCA2a mRNA level preceded the development of cardiac dysfunction (12–15). Furthermore, the cardiac dysfunction was ameliorated dramatically when the decreased level of SERCA2a mRNA was restored (12–14). Previous work from this laboratory has indicated that SERCA2a enzyme activity remained unaltered during the early hyperdynamic stage but was decreased in the late hypodynamic stage of sepsis (16). Because cardiac contractility is regulated by SERCA2a, and SERCA2a gene expression and protein levels were altered under a variety of disease conditions including shock and sepsis (8–16), the present study was undertaken to examine whether the altered expression of SERCA2a is regulated through changes in its gene transcript in the rat heart during the two distinct cardiodynamic phases of sepsis.

Address correspondence to Dr. Maw-Shung Liu, Department of Pharmacological and Physiological Science, St. Louis University School of Medicine, 1402 South Grand Blvd., St. Louis, MO 63104-1028. E-mail: lium@slu.edu.

Address reprint requests to Professor Hseng-Kuang Hsu, Department of Physiology, Kaohsiung Medical University, Kaohsiung, Taiwan.

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MATERIALS AND METHODS

Materials

[α - ^{32}P]dCTP (3000 Ci/mmol) and [α - ^{32}P]UTP (3000 Ci/mmol) were purchased from ICN Pharmaceuticals (Costa Mesa, CA). Tris and SDS were products of Sigma Chemical Co. (St. Louis, MO). Actinomycin D, proteinase K, ribonuclease A, deoxyribonuclease I, and BamHI were supplied by Promega Corp. (Madison, WI). Full-length SERCA2a cDNA from rabbit cloned into the BamHI site of Bluescript plasmid (the insert was 1.98 kb) (17) was a generous gift from Dr. David H. MacLennan, Banting and Best Department of Medical Research, University of Toronto. A monoclonal antibody specific to rabbit SERCA2a was obtained from Affinity Bioreagents, Inc. (Golden, CO). Anti-mouse Ig, horseradish peroxidase-linked species-specific whole antibody, ECL Western blotting detection agent, and Hyperfilm-ECL were products of Amersham Life Science (Arlington Heights, IL). Other chemicals and reagents were of analytic grade.

Animal model

All animal experiments in this study were performed with the approval of the Animal Care Committee of St. Louis University School of Medicine, and in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*. Male Sprague-Dawley rats weighing 270–320 g were used. All animals were fasted overnight with free access to water. They were divided into three groups: control, early sepsis, and late sepsis. Sepsis was induced by cecal ligation and puncture (CLP) as described by Wichterman et al. (18) with minor modification. Under halothane anesthesia, a laparotomy was performed (the size of the incision was 2.5 cm), and the cecum was ligated with a 3-0 silk suture and punctured twice with an 18-gauge needle. The cecum was then returned to the peritoneal cavity, and the abdomen was closed in two layers. Control rats were sham-operated (a laparotomy was performed, and the cecum was manipulated but neither ligated nor punctured). All animals were resuscitated with 4 mL/100 g body weight of normal saline at the completion of surgery and also at 7 h postsurgery. Animals were fasted but had free access to water after operative procedures. After the animals had been anesthetized with 2% chloralose in 20% urethane (5 mL/kg, i.p.), hearts were removed 9 or 18 h postoperation and were then used for the molecular biological studies. It is noteworthy that tissues studied were obtained only from groups surviving to the point of harvest. Early and late sepsis refers to those animals sacrificed at 9 and 18 h, respectively, after CLP. Previous experiments have established that septic rat myocardium was in hyperdynamic state during early phase (9 h post-CLP) but was in hypodynamic state during late phase (18 h post-CLP). In addition, the mortality rates were 0% for control, 11% for early sepsis (measured at 9 h post-CLP), and 19% for late sepsis (measured at 18 h post-CLP) (16, 19, 20). It should be mentioned that the experimental conditions for the sepsis model in current studies was identical to those in previous studies (16, 19, 20).

Determination of SERCA2a protein level by Western blot analysis

Western blot analysis was performed according to the method of Gallagher et al. (21) with minor modification (22). Samples of cardiac homogenates containing 90 μ g protein were denatured and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (7%–10% polyacrylamide gradient gel). Using Bio-Rad Trans-Blot cell, proteins separated by SDS-PAGE were transferred to a polyvinylidene fluoride membrane (Bio-Rad) at 8 mA (constant current) for 14–16 h in transfer buffer [25 mM Tris base, pH 8.3, 192 mM glycine, 5% (vol/vol) methanol]. Nonspecific binding sites were blocked with 10% (wt/vol) of nonfat dry milk in Tris-buffered saline (TBS, 20 mM Tris-HCl, pH 7.4, 137 mM NaCl) for 4 h at 4°C. Blots were washed three times (10 min each) with TBST (TBS containing 0.5% Tween-20) followed by incubation with a 1:400 dilution of a monoclonal antibody raised to rabbit SERCAa for 5 h at room temperature. Subsequently, the blots were washed three times (10 min each) with TBS and then incubated with a 1:1000 dilution of an anti-mouse Ig, horseradish peroxidase-linked species-specific whole antibody for 1 h at room temperature. The blots were then washed four times (10 min each) with TBST, followed by incubation with enhanced chemiluminescent (ECL) Western blotting detection reagent, and finally exposed to Hyperfilm-ECL for 1 h. Autoradiographs were scanned with a Hewlett-Packard ScanJet 4C Scanner, and the relative densities were quantified by a Jandel Scientific Software program (SigmaGel).

Determination of the steady-state level of SERCA2a mRNA by Northern blot analysis

Northern blot analysis was performed as described by Sambrook et al. (23) with modification (22). Total cellular RNA was extracted from control and septic rat hearts with acid guanidinium thiocyanate-phenol-chloroform mixture using a RNA isolation kit (bulletin 1, TEL-TEST "B," Friendswood, TX). RNA concentration was determined by absorbance at 260 nm, and the purity was assayed by the 260/280 nm ratio. Samples containing 30 μ g of total cellular RNA were denatured, size fractionated on 1% agarose-6.6% formaldehyde denaturing gels, and transferred to

nylon membranes (Micron Separations, Westborough, MA) in $10 \times$ SSC (saline-sodium citrate) ($1 \times$ SSC contained 0.15 M NaCl and 15 mM trisodium citrate, pH 7.0) using standard method (23). The membranes were ultraviolet cross-linked, baked at 80°C for 2 h, and then prehybridized at 42°C for 3 h in 50% formamide, $5 \times$ SSC, $5 \times$ Denhart solution [$1 \times$ Denhart solution contains 0.02% each of bovine serum albumin (BSA), polyvinylpyrrolidone, and Ficoll], 0.5% SDS, and 100 μ g/mL of denatured fragmented salmon sperm DNA. The prehybridization solution was replaced with fresh solution containing radioactive cDNA probe labeled with [α - ^{32}P]dCTP according to the random primer labeling technique (23), and the mixture was hybridized overnight at 42°C. The specific cDNA probe used was a full-length SERCA2a cDNA cloned into the BamHI site (5'...3' orientation) of Bluescript plasmid (the insert was 1.98 kb). Insert-containing plasmids were digested with the appropriate restriction enzyme, and the inserts were purified by preparative agarose gel electrophoresis. After hybridization, the membranes were stringently washed and then exposed to PhosphorImager screen. Hybridized [^{32}P]SERCA2a mRNA transcripts were scanned and quantified with PhosphorImager and ImageQuant (Molecular Dynamics, Inc.) and normalized by the input labeled G3PDH cDNA probe.

Measurement of the transcription rate of SERCA2a mRNA by nuclear runoff assay

Cardiac nucleus isolation and nuclear runoff assay were performed by the methods of Boheler et al. (24) and Greenberg et al. (25), respectively, with modification. For the isolation of cardiac nuclei, approximately 2 g of heart tissue was homogenized in 10 vol. of Buffer A [10 mM Tris-HCl, pH 7.4, 10 mM MgCl₂, 0.25 M sucrose, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 2.8 mM dithiothreitol (DTT), 20 units/mL recombinant RNasin], and the homogenate was centrifuged at 1000 \times g for 10 min. The pellet was resuspended in 15 vol of Buffer A, rehomogenized, filtered through a cell strainer (sieve mesh 45 μ m and 70 μ m), and then centrifuged. The resulting pellet was resuspended in Buffer A containing 0.5% Triton X-100 and centrifuged at 1000 \times g for 10 min. The resultant pellet was resuspended in Buffer B (10 mM Tris-HCl, pH 7.4, 10 mM MgCl₂, 0.1 mM PMSF, 2.8 mM DTT, 20 units/mL RNasin, and 2.4 M sucrose), and centrifuged at 52,700 \times g for 2 h. The 52,700 g pellet was resuspended in Buffer A, centrifuged at 1000 \times g for 10 min, and the final pellet containing cardiac nuclei was resuspended in Keller storage buffer (40% glycerol containing 5 mM MgCl₂, 0.1 mM EDTA, 5 mM DTT, and 50 mM Tris-HCl, pH 8.0) to give a final concentration of 1.0×10^7 nuclei per 200 μ L buffer. The cardiac nuclei were frozen in liquid nitrogen, stored at -80°C, and then used for nuclear runoff assay. For nuclear runoff assay, the reaction mixture in a final volume of 0.5 mL contained 20 mM Tris-HCl, pH 8.0, 150 mM KCl, 5 mM MgCl₂, 2 mM DTT, 0.5 mM each of ATP, CTP, and GTP, and 0.2 μ M of [α - ^{32}P]UTP containing a radioactivity of 250 μ Ci. The reaction was initiated by the addition of 1.0×10^7 nuclei, allowed to proceed for 30 min at 30°C, and then terminated by the addition of 200 units of deoxyribonuclease I in 300 mM NaCl, 5 mM MgCl₂, 10 mM Tris-HCl, pH 7.5, and 1.2 mM CaCl₂. Subsequently, 150 μ g of proteinase K in the presence of 1% SDS and 5 mM EDTA were added, and the mixture was incubated at 37°C for 30 min. ^{32}P -labeled RNA was extracted and purified as described by Greenberg et al. (25). The [^{32}P]RNA (about 1.0×10^7 cpm) was hybridized in 1.4 mL of hybridization solution [10 mM N-tris (hydroxymethyl)methyl-2-aminoethanesulfonic acid, pH 7.4, 10 mM EDTA, 0.2% SDS, 300 mM NaCl] with nonlabeled SERCA2a (1.98 kb SERCA2a cDNA cloned into the BamHI site of Bluescript plasmid) and G3PDH cDNA probes, which were immobilized on nitrocellulose membranes. Dot-blot hybridization was carried out at 65°C for 48 h. After hybridization, the membranes were washed three times in $1 \times$ SSC at room temperature, two times in $1 \times$ SSC at 65°C for 30 min, and then incubated with ribonuclease A (10 μ g/mL) at 37°C for 30 min. Following stringent washing, the membranes were exposed to the PhosphorImager screen. Autoradiographs were scanned, and the relative densities were quantified as described earlier. The transcription rate of SERCA2a mRNA was calculated from [^{32}P]RNA bound to the specific cDNA fragment for SERCA2a and corrected for the amount of input labeled RNA from G3PDH cDNA probe.

Stability (half-life) assay of cardiac SERCA2a gene transcript

The stability of heart SERCA2a mRNA was measured by actinomycin D pulse-chase method (26) with modification (22). Heart tissue slices (20–30 mg each) were prepared and incubated at 37°C in Krebs-Henseleit buffer (pH 7.4) containing 2% dialyzed BSA and 5 μ g/mL of actinomycin D for various time intervals (0, 2, 4, and 6 h). The reaction mixture was constantly equilibrated with a stream of 95% O₂-5% CO₂. At the end of each incubation, total RNA was extracted from each sample. An aliquot (30 μ g) of mRNA was dot-blotted on a nylon membrane and hybridized with ^{32}P -labeled cDNA probe (1.98 kb cDNA fragment of rat SERCA2a) as described for Northern blot analysis. Stringent posthybridization washing of the membranes and the detection of the hybridized signals were performed as described above. The half-life of SERCA2a mRNA was defined as the time required for the 50% reduction in mRNA level.

Protein assay and statistical analysis

The protein content of cardiac tissue homogenates were measured as described by Lowry et al. (27). The statistical analysis of the data was performed using one-way ANOVA followed by Student-Newman-Keuls tests. A *P* value of less than 0.05 was considered statistically significant.

RESULTS

Figure 1A shows a representative autoradiograph, and Figure 1B, the quantitative analysis of Western blot for SERCA2a in the rat heart during different phases of sepsis. SERCA2a protein level was unchanged during early sepsis but was decreased by 59% ($P < 0.01$) during late sepsis (Fig. 1B). The sepsis-induced changes in SERCA2a protein concentration were paralleled by changes in SERCA2a Ca^{2+} uptake activity as reported previously by this laboratory (16). These findings demonstrate that SR Ca^{2+} uptake was impaired during the late phase of sepsis, and furthermore, the impairment was a result of the reduction in the protein level of SERCA2a.

Figure 2 shows Northern blot analysis of the steady-state level of SERCA2a mRNA in the control and septic rat hearts. Analyses of the densitometric signals reveal that the steady-state level of SERCA2a mRNA was unchanged during the early sepsis and was decreased by 43% ($P < 0.01$) during late sepsis (Fig. 2B). It should be mentioned that the yields (in mg/g wet wt: 0.54 ± 0.03 , 0.52 ± 0.03 , 0.53 ± 0.04 for control, early sepsis, and late sepsis, respectively) and the purities (A260/280: 1.88 ± 0.03 , 1.86 ± 0.01 , and 1.84 ± 0.02 for control, early sepsis, and late sepsis, respectively) of total RNA remained unaltered among control, early septic, and late septic groups, indicating that changes in the steady-state level of SERCA2a mRNA were not a result of alterations in the isolation procedure

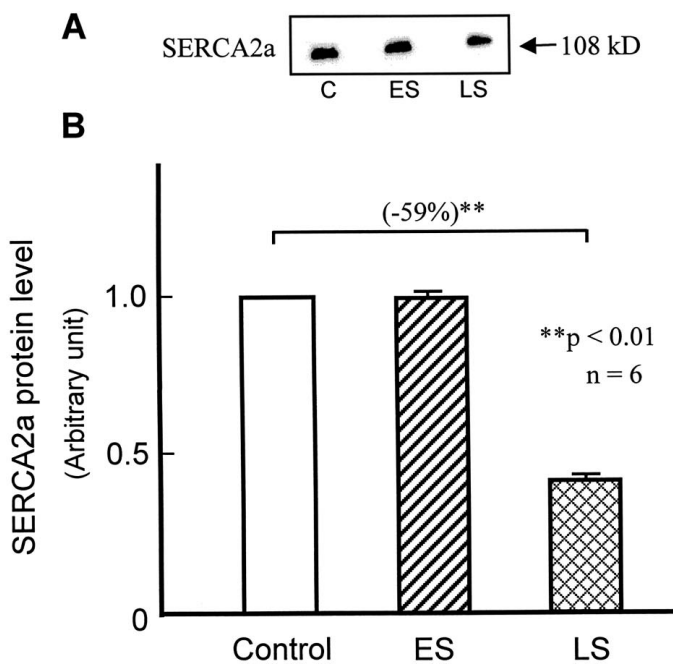


FIG. 1. A, A representative autoradiograph of Western blot analysis. B, Western blot analysis of SERCA2a protein levels in the control and septic rat hearts. Western blot analysis was carried out as described in Materials and Methods using monoclonal antibodies raised to rabbit SERCA2a. Each bar represents the mean \pm SE of six experiments. C, control; ES, early sepsis; LS, late sepsis.

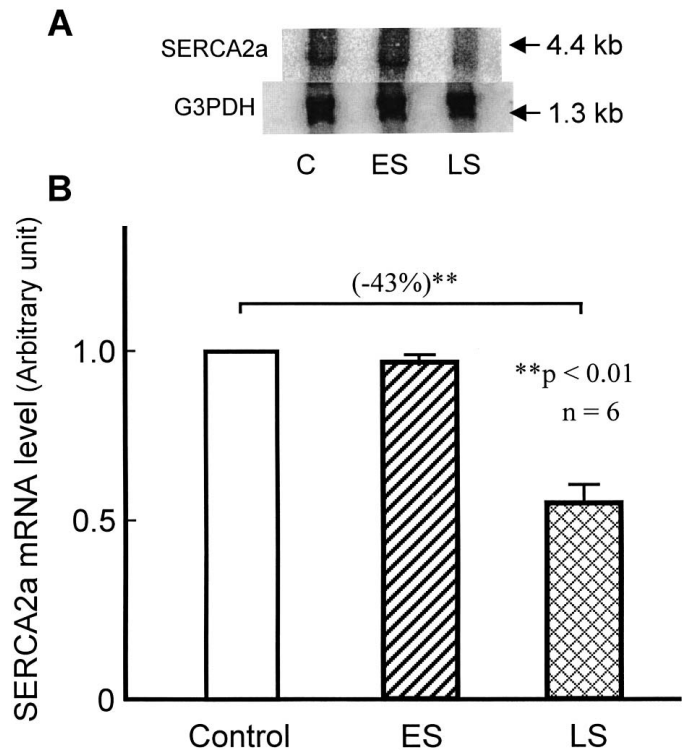


FIG. 2. A, A representative autoradiograph of Northern blot analysis. B, Northern blot analysis of the steady-state level of SERCA2a mRNA in control and septic rat hearts. Northern blot analysis was performed as described in Materials and Methods using total cellular RNA. The specific cDNA probe used was a full-length SERCA2a cDNA (1.98 kb) cloned into the BamHI site of Bluescript plasmid. All SERCA2a mRNA levels were normalized relative to the level of G3PDH to correct for the potential difference in the amount of RNA loaded. C, control; ES, early sepsis; LS, late sepsis.

for cardiac RNA. These results demonstrate that SERCA2a gene transcription in the rat heart was underexpressed during the late phase of sepsis.

Figure 3 depicts changes in the transcription rate of SERCA2a mRNA in the control and septic rat hearts. Nuclear runoff assays reveal that the transcription rate of the SERCA2a gene transcript was unaltered during early sepsis but was decreased by 34% ($P < 0.01$) during the late phase of sepsis (Fig. 3B). It should be mentioned that the yields (in 10^6 nuclei/g wet weight: 11.0 ± 0.7 , 10.3 ± 0.07 , and 10.9 ± 0.7 for control, early sepsis, and late sepsis, respectively) and the viabilities ($94 \pm 1\%$, $92 \pm 1\%$, and $93 \pm 1\%$ for control, early sepsis, and late sepsis, respectively) of cardiac nuclei isolated from control, early septic, and late septic rats were comparable, indicating that changes in the transcription rate of SERCA2a gene transcript, as shown in Figure 3B, were not a result of changes in the isolation procedure for cardiac nuclei. These results demonstrate that the rate of synthesis of SERCA2a mRNA was decreased in the rat heart during the late phase of sepsis.

Figure 4 shows changes in stability of SERCA2a mRNA during the early and the late phases of sepsis. The actinomycin D pulse-chase assays reveal that the half-life of SERCA2a mRNA remained constant during both the early and the late phases of sepsis (Fig. 4B). These data demonstrate that the rate of degradation of SERCA2a mRNA was unaltered in the rat heart during the progression of sepsis.

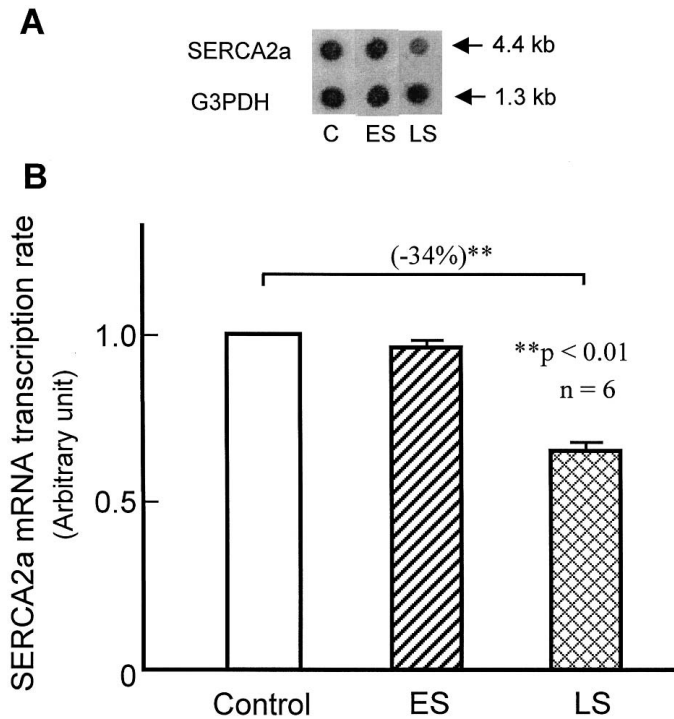


FIG. 3. **A**, A representative autoradiograph of nuclear runoff assay. **B**, Nuclear runoff assay of the transcription rates of SERCA2a mRNA in the control and septic rat hearts. Nuclear runoff assay was conducted as described in the Materials and Methods using SERCA2a cDNA cloned into the BamHI site of Bluescript plasmid and the synthetic G3PDH oligonucleotide as specific probes. C, control; ES, early sepsis; LS, late sepsis.

DISCUSSION

The present data demonstrate that SERCA2a protein level and mRNA abundance were decreased significantly during the late phase of sepsis. Furthermore, decreases in the protein content and the steady-state level of mRNA for SERCA2a during the late phase of sepsis were correlated with the decline in the rate of synthesis of SERCA2a gene transcript. It is of interest to note that the degradation rate (half-life) of SERCA2a mRNA remained unchanged during the progression of sepsis. These results suggest that the decreases in protein expression and mRNA abundance of SERCA2a were a result of the decline in the rate of synthesis of SERCA2a gene transcript. These findings provide a mechanistic explanation for sepsis-induced impairment in SERCA2a protein and gene expression and may contribute to the understanding of the pathogenesis of cardiac dysfunction during the progression of sepsis.

The pathophysiological relevance of the altered SERCA2a gene transcript and protein expression in relation to alteration in cardiac function during the progression of sepsis is obvious. The decreases in SERCA2a activity (16) and mRNA/protein expression (current study) decrease Ca²⁺ uptake into the SR lumen, delay the removal of Ca²⁺ from contractile protein binding, and consequently impede the initiation of the relaxation phase of the contraction-relaxation cycle (4-7). In addition, the decreases in SERCA2a activity and mRNA/protein expression reduce the availability of Ca²⁺ to be released from the SR lumen through the ryanodine-sensitive Ca²⁺ release channel, eventually impairing the activation of the contractile

phase of the contraction-relaxation cycle (4, 5). The final outcome of these events would be a depression in cardiac contractility. This may explain why myocardial contractility was depressed during the late phase of sepsis (3). Our finding that the decreases in SERCA2a protein and gene expression during the late phase of sepsis were the result of the decline in the rate of transcription of SERCA2a gene transcript thus may contribute to the understanding of the molecular pathogenesis of cardiac dysfunction during sepsis. The notion that transcriptional regulation plays a key role in altering SERCA2 gene/protein expression and cardiac function during the late phase of sepsis is further supported by a transgenic mouse study that found that overexpression of SERCA2 gene in the heart leads to an increase in SR Ca²⁺ transport function and increased cardiac contractility (28).

The underlying mechanism responsible for the decrease in the rate of transcription of SERCA2a mRNA in the rat heart during the late phase of sepsis has not been fully explored. There are three isoforms that encode the SR Ca²⁺-ATPase gene: SERCA1, SERCA2, and SERCA3, with SERCA2 being the predominant form expressed in mammalian cardiac muscle (29). The primary SERCA2 gene contains several cryptic exons that allow alternative splicing in its 3' region (29). Primer extension and RNase protection analyses of SERCA2 gene in pressure-overload cardiac hypertrophy have identified

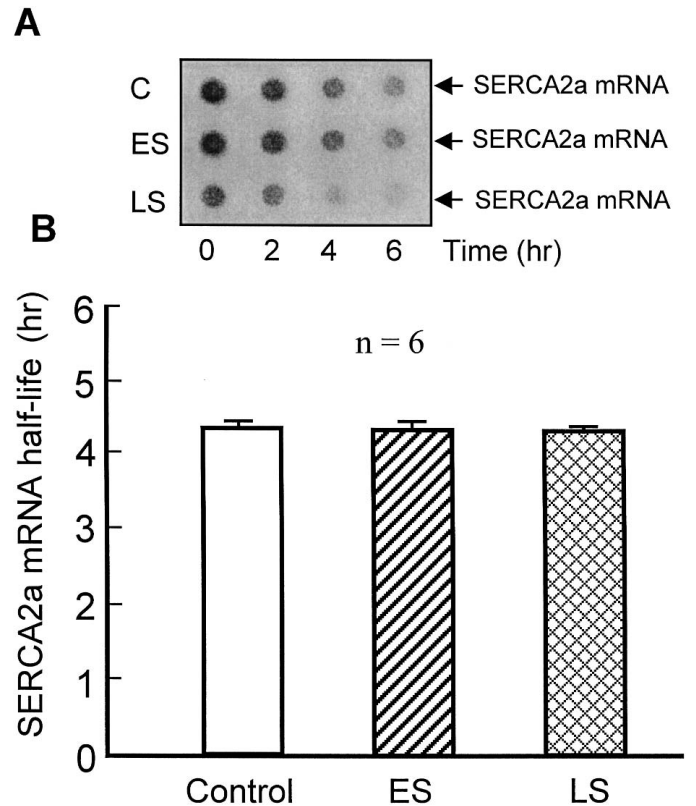


FIG. 4. **A**, A representative autoradiograph of half-life assay. **B**, Stability (half-life) assay of SERCA2a gene transcripts in the control and septic rat hearts. The half-life of SERCA2a gene transcripts was measured as described in Materials and Methods using total cellular RNA prepared from heart tissue slices. The specific cDNA probes used were identical to that of Northern blot analysis. The half-life of SERCA2a gene transcript was calculated as time required for the 50% reduction in mRNA level. C, control; ES, early sepsis, LS, late sepsis.

three major transcription regulatory elements: fragment -658 to -284 bp is the transcription activation site, whereas fragments -1810 to -1110 bp and -284 to -72 bp are the transcription inhibition elements (30). In addition, there are two binding sites for cAMP response element (CRE) and GATA (between -1810 to -1110 bp), four binding sites for SP-1 (between -284 to -72 bp), three binding sites for thyroid hormone (-481 to -458 bp, -310 to -289 bp, and -219 to -195 bp), and two binding sites for myocyte enhancer-specific factor-2 (MEF-2) (within the 5'-flanking region) that are present in the SERCA2 gene. All of these elements may participate in regulating the transcription of SERCA2 gene transcript (30, 31). Cell- or tissue type-specific control of gene transcription requires the availability of a correct set of DNA-binding proteins as transcription factors that associate with DNA response elements. A variety of transcription factors such as CRE binding protein (CREB), MEF-2, T₃Rα1, and Egr-1 have been reported to affect the transcription of cardiac SERCA2 in H9c2 cells, an embryonic heart-derived cell line, on transfection of various factors (31). Because cardiac dysfunction in pressure-overload hypertrophy (30) and heart-derived cell line on cotransfection (31) do not necessarily resemble the pattern of changes in cardiac function in sepsis, further investigation is needed to determine whether any of the reported transfection factors participate in the regulation of the altered SERCA2a gene expression in the rat heart during the late phase of sepsis.

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