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Intracellular Redistribution of Dihydropyridine Receptor in the Rat Heart During the Progression of Sepsis

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Background. **Dihydropyridine receptor (DHPR) regulates the rate and force of cardiac muscle contraction. This study examined the alteration in the intracellular redistribution of DHPR and its association with the development of the two distinct cardiodynamic states in the rat heart during the progression of sepsis.**

Material and methods. **Sepsis was induced by cecal ligation and puncture (CLP). DHPRs were assayed using [^{3}H]PN200-100 binding and photoaffinity labeling with [^{3}H]azidopine followed by polyacrylamide gel electrophoresis.**

Results. **[^{3}H]PN200-110 binding shows that during the early hyperdynamic phase of sepsis (9 h post-CLP), the Bmax was increased by 27% in sarcolemma while decreased by 24% in light vesicle. During the late hypodynamic phase of sepsis (18 h post-CLP), the Bmax was decreased by 39% in sarcolemma but increased by 59% in light vesicle. The sum of the Bmax for both membrane fractions was increased by 16% during early sepsis while decreased by 17% during late sepsis. Photoaffinity labeling shows that the incorporation of [^{3}H]azidopine into 165 kDa peptides during early sepsis was increased by 28% in sarcolemma whereas decreased by 23% in light vesicle. During late sepsis, the incorporation was decreased by 38% in sarcolemma but increased by 46% in light vesicle. The sum of the 165 kDa peptides for both membrane fractions was increased by 13% during early while decreased by 13% during late sepsis.**

Conclusions. **These data indicate that DHPRs in the rat heart were externalized from light vesicles to sarcolemma during the early hyperdynamic phase whereas they were internalized from surface membranes to intracellular sites during the late hypodynamic phase of sepsis. Furthermore, DHPRs were overexpressed during early sepsis while they were underexpressed during late sepsis. Alterations in the expression and intracellular redistribution of DHPRs may contribute to the development of the biphasic cardiodynamic states during the progression of sepsis.**

Key Words: **sarcolemmal membrane; light vesicle; L-type calcium channel; dihydropyridine receptor; receptor externalization; receptor internalization; rat heart; septic shock.**

INTRODUCTION

In the heart, a very low Ca^{2+} concentration (10^{-5}-10^{-7} M) is a prerequisite for the proper excitability and contractility of cardiac muscle cell, and furthermore, the intracellular Ca^{2+} concentration oscillates during systole and diastole [1]. The very low intracellular Ca^{2+} concentration is maintained by a concerted action of dihydropyridine receptor (DHPR) (L-type Ca^{2+} channel) [2], Ca^{2+}-ATPase (PMCA) [3, 4], and Na^{+}-Ca^{2+} exchanger [4] in the sarcolemmal membrane. The oscillation of Ca^{2+} concentration during the systole and diastole is regulated through Ca^{2+}-ATPase (SERCA) [5], ryanodine receptor (Ca^{2+}-induced Ca^{2+} release channel) [5, 6], and phospholamban [5] in the sarcoplasmic reticulum (SR). Progress in the molecular pathogenesis of cardiac diseases such as ischemia/reperfusion injury, hypertrophic cardiomyopathy, and heart failure have provided a cause-and-effect link between the altered dynamics of Ca^{2+} transport proteins and myocardial dysfunction [5–11]. Previous studies from this laboratory have indicated that the dynamics of a number of cardiac Ca^{2+} regulatory proteins including SR Ca^{2+}-ATPase, ryanodine receptor, and phospholamban phos-

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phorylation were altered and the alterations are associated with changes in the contractile state of the myocardium during sepsis [12–14]. The present study dealing with sepsis-induced changes in the intracellular redistribution of DHPR represents part of a systemic investigation regarding roles of Ca^{2+} regulatory proteins on the pathogenesis of altered cardiac function during the progress of sepsis.

MATERIALS AND METHODS

Animal Model

All animal experiments in this study were performed with the approval of the Animal Care Committee of Saint 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 from 270 to 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.* [15] 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 ligature 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 post-surgery. Animals were fasted but had free access to water after operative procedures. Under chloralose and urethane anesthesia, hearts removed from septic or control rats were used for the preparation of sarcolemmal membrane and light vesicle. Early and late sepsis refers to those animals sacrificed at 9 and 18 h, respectively, after CLP. Previous experiments have indicated that septic rats were in the hyperdynamic/hypermetabolic state (cardiac output, heart rate, myocardial $+dP/dt_{max}$, plasma glucose, plasma catecholamines, and body temperature were elevated) during the early sepsis while they were in the hypodynamic/hypometabolic state (cardiac output, heart rate, mean arterial pressure, myocardial $+dP/dt_{max}$, myocardial $-dP/dt_{max}$, plasma glucose, and body temperature were diminished whereas plasma catecholamine levels were elevated) during the late sepsis [16]. The mortality rates were 0% for control, 8% for early sepsis, and 19% for late sepsis.

Preparation of Cardiac Sarcolemmal Membrane and Light Vesicle

Cardiac sarcolemmal membrane and light vesicle were prepared by the methods of Jones [17] and Maisel *et al.* [18] with modification [16, 19]. Hearts removed from septic or control rats were homogenized with Tekmar Tissumizer (Model SDT) in 5 vol of buffer A (50 mM NaH_2PO_4/Na_2HPO_4, pH 7.4, 10 mM EDTA, 25 mM NaF, 1 μg/mL soybean trypsin inhibitor, 1 μg/mL aprotinin, 0.75 μg/mL pepstatin A, and 2 μg/mL leupeptin). The homogenates were centrifuged at 14,000 g for 20 min. The resulting pellets were suspended in buffer B (0.6 M NaCl, 50 mM NaH_2PO_4/Na_2HPO_4, pH 7.4, 10 mM EDTA, and 25 mM NaF), rehomogenized, and recentrifuged at 14,000 $\times$ *g* for 20 min. The pellets from the second centrifugation were suspended in buffer A, homogenized three times (each time for 30 s), and centrifuged at 2,300 $\times$ *g* for 15 min. The resulting pellets were resuspended, rehomogenized, and recentrifuged at 2,300 $\times$ *g* for 15 min. The 2,300 g supernatants were combined and centrifuged at 79,700 $\times$ *g* for 40 min. The 79,700 $\times$ *g* supernatants were further centrifuged at 162,600 g for 40 min and the resulting pellets were suspended in buffer C (0.25 M sucrose and 30 mM histidine, pH 7.4) and then used as light vesicles. The 79,700 $\times$ *g* pellets were suspended in 1 M sucrose dissolved in buffer D (0.3 M NaCl, 50 mM $Na_4P_2O_7$, and 100 mM Tris-HCl, pH 7.4). Ten ml of this suspension were layered at the bottom of a discontinuous sucrose gradient consisting of 9 mL of 0.6 M sucrose (dissolved in buffer D) and 9 mL of 0.25 M sucrose (dissolved in 10 mM histidine, pH 7.4). The gradients were centrifuged at 254,100 $\times$ *g* for 70 min. Fractions at 0.6:0.25 M sucrose interfaces were collected, diluted with buffer D, and then centrifuged at 162,600 $\times$ *g* for 40 min. The final pellets were suspended in buffer C and then used as saracolemmal membranes.

Dihydropyridine Receptor Binding Assay With (+)[^{3}H]PN200-100

DHPR binding assay was carried out using (+)[^{3}H]PN200-100 as a radioligand according to a procedure described by Glossmann and Ferry [20] with slight modification. The standard assay mixture in a final volume of 0.25 mL contained 50 mM Tris-HCl (pH 7.2), 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 1 nM (+)[^{3}H]PN200-110 (86 Ci/mmol), and 0 or 1 μM nifedipine. For Scatchard plot analysis, different concentrations of (+)[^{3}H]PN200-100 ranging from 0.0125 to 2 nM were included in the standard assay mixture. The mixture was preincubated at 30°C for 2 min. The binding assay was subsequently initiated by the addition of sarcolemmal membrane (30 μg protein) or light vesicle (60 μg protein) and allowed to proceed for 60 min at 30°C. At the end of each incubation, the reaction mixture was diluted with 4 mL of ice-cold washing buffer (10 mM Tris/HCl and 6.6% polyethylene glycol 6000, pH 7.4) and filtered immediately through a 0.45-μm glass fiber filter paper (Baxter Healthcare, Deerfield, IL) under suction. The filter paper was washed three times with 4 mL of washing buffer, dried, and the radioactivity was then determined with a liquid scintillation counter. The specific binding was defined as the bound radioactivity displaceable by 1 μM nifedipine. The non-specific binding was less than 10% of the total binding.

Photoaffinity Labeling of Dihydropyridine Receptor With [^{3}H]azidopine, and Sodium Dodecylsulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Photoaffinity labeling of cardiac DHPR was performed by the method of Ferry *et al.* [21] with modification. Cardiac sarcolemmal membranes (100 μg protein) or light vesicles (150 μg protein) were incubated in the dark in Sorvall-SS polycarbonate tubes in 1 mL of N_2-saturated buffer E (50 mM Tris-HCl, pH 7.4, 0.1 mM PMSF, and 1 μM pepstatin A) containing 5 nM [^{3}H]azidopine (45 Ci/mmol) and 0 or 2 μM nifedipine for 60 min at 25°C. At the end of incubation, the mixture was centrifuged at 35,000 $\times$ *g* for 10 min at 2°C, and the resulting pellets were resuspended in 10 mL of ice-cold buffer E supplemented with 0.25 mg/mL bovine serum albumin. The centrifugation was repeated twice. The pellets containing cardiac membranes were then photolyzed with Spectroline software lamp for 10 min. The photolyzed samples were spun down as described above and the resulting pellets were suspended in [40 μL of buffer E. Ten μL of a protease inhibitor mixture [40 mM EDTA, 0.4 mg/mL leupeptin, 6 mg/mL trypsin inhibitor, 4 mg/mL PMSF, 4 mg/mL benzamidine, 40 μg/mL pepstatin A, and 0.04 mg/mL Trasylol (Bayer Pharmaceuticals Corp., West Haven, CT)] was added. After 20 min, 50 μL of boiling stop solution (10% SDS, 10% β-mercaptoethanol, 20% glycerol, 125 mM Tris-HCl, pH 7.5, 30% urea, 0.1 mM PMSF, and 0.002% bromphenol blue) was added, and the samples were allowed to stand at room temperature for 10 min. The covalent incorporation of radioligand was analyzed by SDS-PAGE (12% acrylaminde gel) and the electrophoresis was performed as described elsewhere [16]. For the quantification of ^{3}H labeling, the dried gel tracks were cut into 3-mm slices and the radioactivity was determined by liquid scintillation counting.