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Increased intracellular Ca^{2+}: A critical link in the pathophysiology of sepsis?

(dantrolene/septic shock/nuclear magnetic resonance)

SHENG-KWEI SONG*, IRENE E. KARL†, JOSEPH J. H. ACKERMAN‡, AND RICHARD S. HOTCHKISS*§

*Department of Anesthesiology, Research Unit, and †Department of Medicine, Washington University School of Medicine, St. Louis, MO 63110; and ‡Department of Chemistry, Washington University, St. Louis, MO 63130

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ABSTRACT **Severe bloodstream-borne infection—i.e., sepsis—and the resulting multiorgan failure are now the most common cause of death in many intensive care units. One of the most fundamentally important and controversial issues concerning the pathophysiology of sepsis is the role of intracellular free calcium concentration ($[Ca^{2+}]_i$) in this disorder. Because of the critical role of calcium as an intracellular second messenger and as a potential cellular toxin, resolution of this issue is crucial. Using ^{19}F NMR spectroscopy and the calcium indicator 5,5′-difluorobis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetate we demonstrate in the intact perfused organ, the rat thoracic aorta, that $[Ca^{2+}]_i$ in aortic smooth muscle is increased >2-fold during sepsis. Furthermore, we determined that sodium dantrolene, a drug that decreases release of calcium from the sarcoplasmic reticulum and that is lifesaving in malignant hyperthermia (a disorder due to increased $[Ca^{2+}]_i$), is able to reduce the elevated $[Ca^{2+}]_i$ in sepsis to control values when added *in vitro* or when given *in vivo* to the animal. These results suggest that an increase in $[Ca^{2+}]_i$ is an early event in sepsis and that increased $[Ca^{2+}]_i$ may be responsible for, or contribute to, cellular injury. Dantrolene may offer a therapeutic strategy in the treatment of sepsis.**

The most common cause of death in surgical intensive care units in the United States is shock resulting from the systemic response to serious bloodstream-borne infection, a condition commonly described as septic shock (1). A common cause of death during sepsis and a hallmark of the disorder is shock refractory to conventional therapy of volume administration and catecholamine infusions (1). Utilizing animal models of sepsis/endotoxemia, investigators have demonstrated a number of abnormalities in the rat thoracic aorta that, if present in the systemic vasculature, may be responsible for, or contribute to, the refractory shock. The major abnormalities reported in the aorta included decreased contractile response to phenylephrine and KCl (2), decreased α_1 receptor density (3), decreased ability of norepinephrine to activate the inositol phospholipid pathway (3), and decreased $^{45}Ca^{2+}$ influx in aortic microsomal vesicles (4). Although the aortic contractile abnormalities and the changes in cellular and microsomal $^{45}Ca^{2+}$ fluxes have led investigators to conclude that a major effect of sepsis/endotoxemia is an alteration of intracellular calcium homeostasis in vascular smooth muscles, there is no consensus as to the ultimate effect of sepsis on intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) (5). Also, it is possible that sepsis induces no change in $[Ca^{2+}]_i$ and that decreased aortic contractility is due to other mechanisms, such as defects in receptors, in the signal transduction pathway, or in the contractile apparatus itself. No direct measurements of $[Ca^{2+}]_i$ during sepsis/endotoxemia have been performed on vascular smooth muscle cells and there is debate concerning whether $[Ca^{2+}]_i$ is increased or decreased. Given the critical role of calcium as a regulator of numerous cellular processes and its toxic effects when increased excessively, determination of the effect of sepsis on $[Ca^{2+}]_i$ is critically important on the pathogenesis of sepsis and on current and potential therapy of the disorder. The purpose of this study was to determine the effects of sepsis on $[Ca^{2+}]_i$ in an intact perfused organ, the rat thoracic aorta, which is likely to reflect the *in vivo* state. Using ^{19}F NMR spectroscopy and the calcium indicator 5,5′-difluorobis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetate (5FBAPTA), our findings demonstrate that $[Ca^{2+}]_i$ in aortic smooth muscle is increased >2-fold during sepsis. In addition, we have determined that sodium dantrolene, an agent that decreases release of Ca^{2+} from the sarcoplasmic reticulum, is able to reduce the elevation of $[Ca^{2+}]_i$ noted in sepsis to control values when given either *in vitro* or *in vivo* to the animal. These results suggest that an increase in $[Ca^{2+}]_i$ is an early event occurring within the first 24 hr of the septic process and that agents that decrease $[Ca^{2+}]_i$ may be beneficial.

MATERIALS AND METHODS

Materials. 5FBAPTA was purchased from Molecular Probes (Eugene, OR). Sodium dantrolene (Proctor & Gamble) was purchased from the hospital pharmacy as the commercially available preparation used clinically. All other materials were purchased from Sigma.

Animal Model and Aortic Perfusion. Male Sprague–Dawley rats weighing 325–450 g were purchased from Sasco (Omaha, NE). The cecal ligation and perforation model as described by Wichterman *et al.* (6) was used to induce peritonitis. All animal procedures were approved by the Committee on the Humane Care of Laboratory Animals at Washington University. After surgery, animals were kept fasted but allowed free access to water. At 24 hr after surgery, mortality in the septic and sham-operated rats was 15% versus 0%, respectively. Animals were anesthetized with halothane and injected i.v. with heparin (100 units), and a 500-μl blood sample was obtained from the inferior vena cava for analysis of blood lactate according to the methods of Lowry and Passonneau (7). Rats were confirmed to be septic both by elevated blood lactate and by examination of the intraabdominal cavity, which revealed the presence of foul-smelling purulent fluid and a grey-black cecum.

For experiments examining the *in vivo* effect of dantrolene on $[Ca^{2+}]_i$, a dantrolene dose of 3 mg per kg of body weight was injected i.p. immediately following cecal ligation and perforation. A second identical dose of dantrolene was administered i.p. to the septic rats 12 hr later.

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Abbreviations: $[Ca^{2+}]_i$, intracellular $[Ca^{2+}]$; 5FBAPTA, 5,5′-difluorobis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetate.

§To whom reprint requests should be addressed.

A segment of the aorta extending from below the left subclavian artery to the diaphragm (4–5 cm long) was rapidly excised and placed in cold medium on ice. The aorta was trimmed of visible fat and other nonadventitial perivascular tissue. A perfusion cannula was placed into the proximal portion of the aorta and the distal end of the aorta was ligated. The perfusate flows out of the aorta via the small stumps of the branches of the intercostal arteries. The single aorta was placed inside a 5-mm (outer diameter) NMR tube and perfused with Krebs–Henseleit buffer (pH 7.4) containing 5 mM glucose, 3% bovine serum albumin, and 2.5 mM $CaCl_2$ according to the technique of Crass *et al.* (8) and as modified by Jelicks and Gupta (9) for examination via high-resolution NMR spectroscopy. The medium, gassed with 95% O_2/5% CO_2, was perfused at a constant flow rate of 6 ml/min at 37°C.

The effect of dantrolene added to the perfusate on $[Ca^{2+}]_i$ was determined on a subgroup of perfused aortas. After basal $[Ca^{2+}]_i$ was determined in aortas from seven septic and six control rats, 30 μM dantrolene was added to the perfusate and $[Ca^{2+}]_i$ was measured again.

NMR Experiments. NMR studies on perfused rat aorta were performed on a Varian Unity 600 spectrometer operating at 564.4 MHz for ^{19}F NMR data acquisition. All experiments were conducted at 37°C with a 5-mm indirect detection probe and observing through a retuned inner ^1H coil. The ^{19}F NMR spectra were acquired with a pulse repetition time of 0.2 sec, a sweep width of 50 kHz, 5000 complex data points, and a 45° pulse width of 8.5 μsec. After a 30-min recovery period, the membrane-permeant acetoxymethyl ester of 5FBAPTA was added to the perfusion medium at a concentration of 30 μM and ^{19}F NMR data acquisition was begun. There were no differences in the rate of loading of the 5FBAPTA and both control and septic aortas required ≈2 hr to load sufficient 5FBAPTA to permit detection of the ^{19}F resonances. All spectra were acquired while continuously loading the cell-permeant 5FBAPTA acetoxymethyl ester. After loading the 5FBAPTA, no difference in the ratio of the bound to free 5FBAPTA resonances occurred over the next 2–4 hr after loading of the calcium indicator, and therefore $[Ca^{2+}]_i$ remained constant throughout the experimental period. All time-domain free induction decay NMR data were analyzed by Bayesian probability methods developed in this laboratory (10–12) to estimate the ^{19}F NMR resonance amplitudes of the free—i.e., unbound—5FBAPTA and the bound 5FBAPTA—i.e., 5FBAPTA complexed to Ca^{2+}. The $[Ca^{2+}]_i$ was determined by multiplying the amplitude ratio of Ca–5FBAPTA to 5FBAPTA by the dissociation constant (K_d) of the equilibrium reaction between free and bound 5FBAPTA (K_d = 245 nM). Our determination of K_d is in reasonable agreement with Marban *et al.* (13), and the use of K_d values reported by others would change only the derived absolute concentration of $[Ca^{2+}]_i$ but not the relative differences between comparative measurements.

The NMR signal is a volume—i.e., tissue-averaged—signal and therefore is derived from large numbers of cells (13, 14). In the present experiments, the $[Ca^{2+}]_i$ determined by NMR represents the average $[Ca^{2+}]_i$ contained in a 2-cm segment of the aorta. To evaluate the contribution of the different cell types to the NMR signal and to determine possible effects of sepsis on cell type or cell proportion, aortas from a separate group of four identically treated septic and four sham-operated rats were examined by routine histologic section and by transmission electron microscopy. No qualitatively significant differences in septic vs. control aortic samples were demonstrated by either microscopic method. Only two cell types were observed. A single layer of endothelial cells (constituting only a tiny fraction of the cells in the aortic specimen) was present and the remaining vast majority of cells were all smooth muscle cells. No fibroblasts were observed but a large amount of elastin fibers were present throughout all samples.

To confirm the metabolic viability of the perfused aortas, aortas from five septic and seven control rats were freeze-clamped at the end of the ^{19}F NMR data acquisition with tongs precooled in liquid nitrogen. These aortas were extracted with perchloric acid and analyzed for ATP, lactate, and pyruvate according to the methods of Lowry and Passonneau (7). Protein was measured according to the method of Lowry *et al.* (15).

Statistical Analysis. The significance of difference test between two samples of data was carried out by using the RS/1 system from BBN software. The paired *t* test was performed on the data measured from the same animal—i.e., $[Ca^{2+}]_i$ measured before and after administration of sodium dantrolene. For unpaired data, an *F* test was first used to test for the equality of variance. The pooled variance *t* test was used for data with equal variances. The unpooled variance *t* test was used for data with unequal variances. Statistical significance was accepted at the 95% confidence limit. Data are presented as means ± SEM.

RESULTS

Whole blood lactate from 14 septic and 14 control rats revealed the characteristic increase in lactate noted in septic rats (16); lactate was increased >2-fold: 1.9 ± 0.15 vs. 0.88 ± 0.35 mM for septic and control rats, respectively ($P < 0.01$). Analysis of tissue extracts demonstrated no differences in the concentrations of ATP, which were 4.2 ± 0.4 and 4.2 ± 0.3 μmol per g of protein for septic and control aortas, respectively. These concentrations of ATP are comparable to values reported for vascular smooth muscle for other animal species (17). The lactate to pyruvate ratio, a sensitive indicator of cellular hypoxia, was normal in both septic and control aortas (12.09 ± 4.33 and 10.45 ± 2.21, respectively).

Results from ^{19}F NMR spectroscopy demonstrated that sepsis caused $[Ca^{2+}]_i$ to increase >2-fold—i.e., 362 ± 25 nM vs. 151 ± 16 nM in septic and control aortas, respectively ($P < 0.001$) (Fig. 1). Fig. 2 demonstrates a typical ^{19}F NMR spectrum of perfused control rat aorta with $[Ca^{2+}]_i$ equal to 93 nM, perfused septic aorta with $[Ca^{2+}]_i$ equal to 337 nM, and the same septic aorta after addition of 30 μM dantrolene added to the perfusate with $[Ca^{2+}]_i$ reduced to 99 nM. Addition of dantrolene to the perfusion medium caused a marked reduction of the elevated $[Ca^{2+}]_i$ in aortas of seven septic rats (367 ± 29 to 128 ± 22 nM; $P < 0.001$) (Fig. 3). In

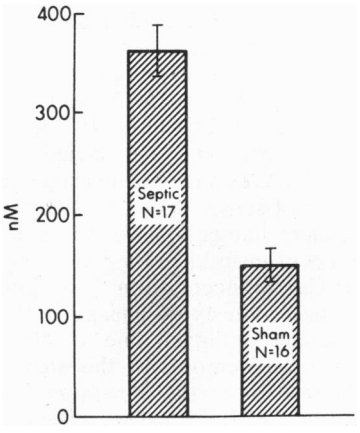

FIG. 1. Comparison of $[Ca^{2+}]_i$ in septic vs. control rat aortas. A total of 17 septic and 16 control rats were examined; $[Ca^{2+}]_i$ was 362 ± 25 and 151 ± 16 nM, respectively ($P < 0.001$). Values are expressed as means ± SEM. An unpaired *t* test was used to compare data from septic vs. control rats.

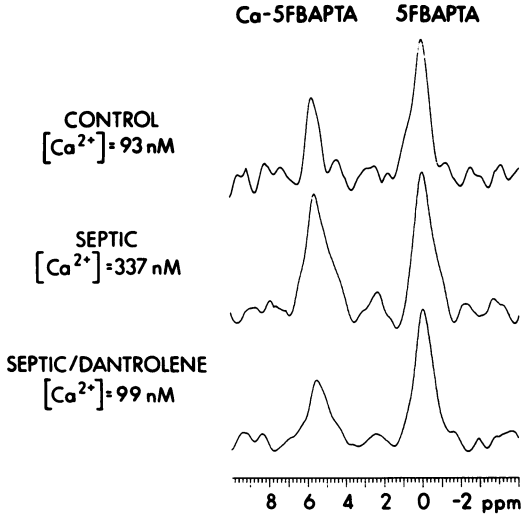

FIG. 2. Typical ^{19}F NMR spectra. Top trace, perfused control rat aorta with $[Ca^{2+}]_i$ equal to 93 nM; middle trace, perfused septic aorta with $[Ca^{2+}]_i$ equal to 337 nM; bottom trace, the same septic rat aorta as in the middle trace acquired after addition of 30 μM sodium dantrolene. The postdantrolene $[Ca^{2+}]_i$ was equal to 99 nM.

six control aortas, dantrolene added to the perfusion medium caused a smaller (17%) and not statistically significant decrease in $[Ca^{2+}]_i$ ($P > 0.05$). Importantly, dantrolene also demonstrated the ability to reduce the $[Ca^{2+}]_i$ present in the septic aortas when it was administered *in vivo* by i.p. injection. The $[Ca^{2+}]_i$ in the aortas of four septic rats that were treated *in vivo* with dantrolene was 150 ± 14 nM (Fig. 3).

DISCUSSION

Although calcium generally acts as an important intracellular second messenger, increased cytosolic calcium can cause cellular toxicity and cell death by activating a number of enzymes that cause degradation of intracellular proteins, cellular membranes, and nuclear DNA (18–20). There is indirect evidence that $[Ca^{2+}]_i$ is elevated in sepsis. Investigators have determined that several calcium-activated enzymes—i.e., calpain (a calcium-activated protease) (21) and glycogen phosphorylase b kinase (22)—have increased activities in tissues of septic animals. The present results demonstrating a marked increase in $[Ca^{2+}]_i$ in aortic smooth muscle during sepsis in an intact organ preparation may reflect the condition noted *in vivo*. If the increased $[Ca^{2+}]_i$ in aortic smooth muscle occurs in cells in other organs, it is possible that a sustained increase in $[Ca^{2+}]_i$ could be playing a major role in the multiple organ failure of sepsis by activation of proteases, phospholipases, endonucleases, and other enzymes (19).

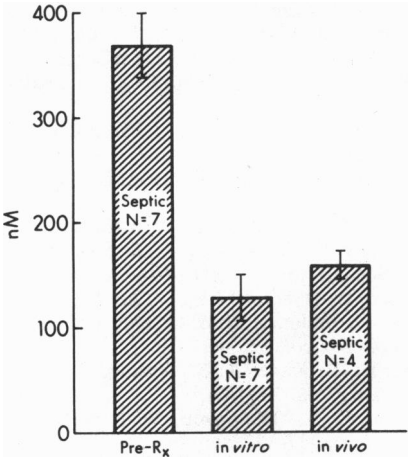

FIG. 3. Effect of dantrolene on $[Ca^{2+}]_i$. After basal $[Ca^{2+}]_i$ was determined as described, seven septic rat aortas were treated by adding 30 μM dantrolene to the perfusate and $[Ca^{2+}]_i$ was measured again, pre-R_x and *in vitro*. A dramatic effect of sodium dantrolene to reduce $[Ca^{2+}]_i$ in the septic rat aortas was observed (367 ± 29 to 128 ± 22 nM; $P < 0.001$; paired t test). A separate group of septic rats received two doses of dantrolene (3 mg/kg) *in vivo* prior to removal and perfusion of the aorta. $[Ca^{2+}]_i$ in the perfused aorta in this group was 150 ± 14 nM and comparable to values for control aortas.

Because the NMR signal represents a tissue-averaged measurement, we cannot definitely exclude the possibility of microheterogeneity of $[Ca^{2+}]_i$ in cells in the septic aorta—i.e., a subpopulation of smooth muscle cells has a greatly increased $[Ca^{2+}]_i$, whereas the bulk of the cells have a $[Ca^{2+}]_i$ comparable to that of control smooth muscle cells. However, none of our findings—i.e., the metabolic data or the electron microscopic examination—provide evidence directly in support of this hypothesis, and thus we believe that this possibility is unlikely. A second potential reason for the difference in the $[Ca^{2+}]_i$ in septic vs. control aortas could be that the septic aortic cells are actively excreting deesterified 5FBAPTA into the extracellular space, giving a falsely elevated signal because of the large concentration of extracellular Ca^{2+}. The fact that the $[Ca^{2+}]_i$ determined in the aortas remained stable for 2–4 hr argues strongly against this possibility. In addition, the effect of dantrolene, which acts on the sarcoplasmic reticulum to lower $[Ca^{2+}]_i$, confirms that 5FBAPTA was reflecting the changes in $[Ca^{2+}]_i$ and not extracellular $[Ca^{2+}]$.

Previous investigations of the effect of sepsis on $[Ca^{2+}]_i$ have not been consistent. Although Deaciuc and Spitzer (23) found that $[Ca^{2+}]_i$ was decreased by ≈40% in hepatocytes isolated from endotoxin-treated compared to control rats, Sayeed and Maitra (24) reported that $[Ca^{2+}]_i$ was increased >3-fold in isolated hepatocytes obtained from endotoxic-treated rats compared to hepatocytes of control rats. Recently, Portoles *et al.* (25) reported that $[Ca^{2+}]_i$ was increased from 123 ± 6 nM in control hepatocytes to 267 ± 8 nM in hepatocytes incubated with 200 μg of endotoxin. Finally, in HEp-2 cells, Baldwin *et al.* (26) found that certain strains of enteropathogenic *Escherichia coli* (but not all of the strains tested) increased $[Ca^{2+}]_i$ from 54 ± 2.0 to 322 ± 70.2 nM. Because $[Ca^{2+}]_i$ determined in isolated cells may not reflect $[Ca^{2+}]_i$ *in vivo*, the present results utilizing ^{19}F NMR and intact perfused tissue represent a major contribution to the current debate about the effect of sepsis on $[Ca^{2+}]_i$. There is recent evidence indicating that $[Ca^{2+}]_i$ may be increased in septic patients. In a preliminary report, erythrocyte $[Ca^{2+}]_i$ was examined in blood samples from septic and nonseptic surgical patients (27). The $[Ca^{2+}]_i$ in the erythrocytes obtained from septic patients was increased compared to values for nonseptic patients—96.3 ± 7.5 and 43.4 ± 2.9 nM, respectively (27).

Our findings relate to the current controversy surrounding the role of calcium administration in patients with sepsis (28). Patients with sepsis frequently have an unexplained decrease in the plasma ionized calcium concentration ($[Ca^{2+}]_e$), with no physiologic explanation for this decreased $[Ca^{2+}]_e$. Our results may indicate that calcium supplementation to these patients (in an attempt to correct the low $[Ca^{2+}]_e$) could be detrimental by exacerbating the intracellular calcium overload. A possible explanation for the increased $[Ca^{2+}]_i$ in the aortic smooth muscle cells may be due to a net movement of extracellular calcium ions into the cells and thus calcium administration would worsen this trend. Additional evidence supporting our concern about administration of calcium to patients with sepsis has been provided by Zaloga *et al.* (29), who reported that calcium administration to septic rats

greatly worsened mortality and caused an increase in circulating plasma endotoxin concentration.

Dantrolene is a drug that has been remarkably effective in virtually eliminating mortality in patients with the highly lethal malignant hyperthermia syndrome, a disorder characterized by increased $[Ca^{2+}]_i$ due to defective handling of Ca^{2+} by the sarcoplasmic reticulum (30). In contrast to calcium-channel blockers, dantrolene does not block calcium channels but rather is thought to reduce $[Ca^{2+}]_i$ via its action to decrease release of Ca^{2+} from the sarcoplasmic reticulum (31). Using ion-specific electrodes, Lopez and colleagues (32) examined the effects of dantrolene on myoplasmic free $[Ca^{2+}]_i$ *in vivo* in normal subjects and in patients susceptible to malignant hyperthermia (MH). The $[Ca^{2+}]_i$ in skeletal muscle was 0.112 ± 0.022 and 0.485 ± 0.022 μM in normal and MH-susceptible patients, respectively. Dantrolene caused a dose-dependent reduction in $[Ca^{2+}]_i$ in MH-susceptible patients to 0.092 ± 0.008 μM. In normal subjects, dantrolene also caused a dose-dependent reduction in $[Ca^{2+}]_i$, lowering it by $\approx$50% to 0.068 ± 0.002 μM at the highest administered dose. Previously, the effects of dantrolene to reduce $[Ca^{2+}]_i$ were thought to be restricted to cells in which the intracellular calcium storage organelle is the sarcoplasmic reticulum. Recently, dantrolene also has been demonstrated to modulate calcium homeostasis in cells in which the intracellular calcium storage organelle is the endoplasmic reticulum. Dantrolene has been demonstrated to reduce resting $[Ca^{2+}]_i$ and to prevent the increase in $[Ca^{2+}]_i$ that occurs from a variety of noxious stimuli in liver (33), brain (34), and osteoblast (35) cells.

A final implication of our study is that agents that lower $[Ca^{2+}]_i$ may be effective in improving survival in patients with sepsis. In studies conducted in our laboratory with incubated epitrochlearis muscle, protein breakdown was increased 2-fold in muscles of septic vs. control rats. Dantrolene either given i.p. to animals or added to the incubation medium reduced the 2-fold increase in the protein degradation noted in septic muscles to values comparable to that of control muscles. In addition, dantrolene caused several of the abnormal blood metabolite concentrations in the septic animals (lactate, ketone bodies, tyrosine) to return to values similar to those of control animals. In plasma from these septic rats, dantrolene reduced lactate from 4.0 ± 0.18 to 2.19 ± 0.15 mmol per liter ($n = 5$) ($P < 0.01$). Dantrolene had a much smaller and not statistically significant effect on plasma of control rats—i.e., 1.99 ± 0.15 and 1.78 ± 0.17 for control and control plus dantrolene, respectively (unpublished data). In support of our findings indicating the efficacy of dantrolene in reducing $[Ca^{2+}]_i$ during sepsis, Baldwin *et al.* (26) reported that dantrolene was able to prevent pathogenic *E. coli* from increasing $[Ca^{2+}]_i$ in HEp-2 cells. It is interesting that the results of Baldwin *et al.* in HEp-2 cells are similar to our findings in aortic smooth muscle cells. It is also important to note that dantrolene was effective in preventing the increased $[Ca^{2+}]_i$ when administered *in vivo* to the septic rats, thus supporting its potential clinical role. Preliminary experiments from our laboratory suggest that dantrolene administered concurrently with endotoxin improved survival in mice (unpublished observations).

Mortality from the sepsis syndrome has not changed significantly over the past several decades and in many intensive care units less than half of the patients with sepsis survive. Given this setting, we believe that the present results together with previous studies demonstrating beneficial effects of calcium-channel blockers indicate that carefully controlled clinical studies with $[Ca^{2+}]_i$ reducing agents such as dantrolene are needed. Other investigators (29) have also called for clinical studies with calcium-channel blockers in septic patients. If increased $[Ca^{2+}]_i$ is determined to be a major cause of morbidity and mortality in sepsis, we speculate that dantrolene may be more beneficial than calcium-channel blockers in treating increased $[Ca^{2+}]_i$ in sepsis because it will be more effective in reducing $[Ca^{2+}]_i$ and because dantrolene (as opposed to calcium-channel blockers) works systemically in a variety of cell types. In support of our contention that dantrolene may be more effective than calcium-channel blockers, it has recently been reported that dantrolene significantly lowers the elevated serum creatine kinase levels in patients with myopathies, presumably by decreasing the activity of calcium-activated proteases (36). On the other hand, calcium-channel blockers have not been effective in this disorder. To end on a word of caution, however, no animal models of sepsis reliably reproduce the sepsis syndrome that occurs in human patients. Also, studies examining therapy of sepsis with cytokine blockers have discovered the "two-edged sword" nature of cytokines. Similarly, an increase in $[Ca^{2+}]_i$ could have beneficial as well as adverse effects on the cell.

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