Lysozyme, a mediator of sepsis, impairs the cardiac neural adrenergic response by nonendothelial release of NO and inhibitory G protein signaling

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Mink SN, Cheng ZQ, Bose R, Jacobs H, Kasian K, Roberts DE, Santos-Martinez LE, Light RB. Lysozyme, a mediator of sepsis, impairs the cardiac neural adrenergic response by nonendothelial release of NO and inhibitory G protein signaling. Am J Physiol Heart Circ Physiol 293: H3140–H3149, 2007. First published August 31, 2007; doi:10.1152/ajpheart.00502.2007.—We previously showed that lysozyme (Lzm-S), derived from leukocytes, caused myocardial depression in canine cepins by binding to the endocardial endothelium to release nitric oxide (NO). NO then diffuses to adjacent myocytes to activate the cGMP pathway. In a canine right ventricular trabecular (RVT) preparation, Lzm-S also decreased the inotropic response to field stimulation (FSR) during which the sympathetic and parasympathetic nerves were stimulated to measure the adrenergic response. In the present study, we determined whether the pathway by which Lzm-S decreased FSR was different from the pathway by which Lzm-S reduced steady-state (SS) contraction. Furthermore, we determined whether the decrease in FSR was due to a decrease in sympathetic stimulation or enhanced parasympathetic signaling. In the RVT preparation, we found that the inhibitory effect of Lzm-S on FSR was prevented by NO synthase (NOS) inhibitors. A cGMP inhibitor also blocked the depressant activity of Lzm-S. However, in contrast to the Lzm-S-induced decline in SS contraction, chemical removal of the endocardial endothelium by Triton X-100 to eliminate endothelial NO release did not prevent the decrease in FSR. An inhibitory G protein was involved in the effect of Lzm-S, since FSR could be restored by treatment with pertussis toxin. Atropine prevented the Lzm-S-induced decline in FSR, whereas β1- and β2-adrenoceptor function was not impaired by Lzm-S. These results indicate that the Lzm-S-induced decrease in FSR results from a nonendothelial release of NO. NO then acts through inhibitory G protein to enhance parasympathetic signaling.

adrenergic β-receptors; septic shock; myocardial depression; sympathetic response

Septic shock is a clinical syndrome that results from an activated systemic host inflammatory response to infection, leading to cardiovascular collapse (2). Reversible myocardial depression is an important component of the circulatory collapse that develops in septic shock as a result of the release of inflammatory mediators (32, 33). Among the physiological compensatory mechanisms that would potentially limit loss of cardiac function in this condition, stimulation of the cardiac sympathetic nerves, which leads to an increase in the release of norepinephrine (NE), would be expected to be important. In a previous study, we used pore filtration and mass spectroscopy—mass sequencing (MS-MS) to identify a myocardial depressant factor that developed after Escherichia coli infusion in a canine model of septic shock (18, 27–29). We found that the inflammatory mediator lysozyme (Lzm-S), released from neutrophils and macrophages (5, 10, 16), contributed to the development of myocardial dysfunction in this model. Moreover, we found that, in addition to reducing steady-state (SS) contraction, Lzm-S attenuated the response to field stimulation (FSR) in a canine right ventricular trabecular (RVT) preparation in which the sympathetic and parasympathetic nerves were stimulated (28). In subsequent experiments, we demonstrated that the mechanism by which Lzm-S decreased SS depression was mediated by nitric oxide (NO) release (27). We showed that Lzm-S interacted with the cardiac endothelium by binding to an N-glycoprotein to cause the release of NO (18, 27). NO then diffused to adjacent myocytes to activate the NO-cGMP pathway, which, in turn, led to myocardial depression (27).

The pathway by which Lzm-S may lead to a decrease in FSR is not clear. Whereas this pathway may be identical to the pathway that reduces a decline in SS contraction, the cardiac neurobiology of NO synthases (NOS) is complex (8, 25). In addition to the paracrine actions of NO that involve NO release by the endocardial endothelium (EE), there are multiple sources of NO in the autonomic nerves and cardiomyocytes, all of which might be potential regulators of cardiac excitability and cell signaling in response to field stimulation (8, 25). Moreover, the net cardiac FSR is determined by the interaction between the sympathetic and parasympathetic systems, in which the predominant contribution of the sympathetic system leads to the usually observed increase in contractility representing the adrenergic response (8, 25).

In the present study, we determined whether the Lzm-S-induced reduction in the adrenergic FSR was mediated by NO-cGMP signaling and whether NO release was derived from EE or myocyte/autonomic nerves. We also determined whether this dysfunction was related to a decrease in sympathetic neural release of NE or an increase in neuronal parasympathetic signaling.

METHODS

The experiments were approved by the University Animal Care Committee and conform with the National Institutes of Health guidelines for the care and use of laboratory animals (30).

The RVT preparation has been described previously (7, 13, 22, 28). Briefly, mongrel dogs (15–25 kg body wt) were anesthetized with...
pentobarbital sodium. The hearts were removed, flushed intraluminally with 50 ml of Krebs-Henseleit (KH) solution (in mM: 118 NaCl, 4.7 KCl, 2.5 CaCl₂, 1.2 MgSO₄, 1.4 KH₂PO₄, 25 NaHCO₃, and 11 dextrose), and placed in ice-cold KH solution bubbled with 95% O₂-5% CO₂. Three to four thin (<1-mm-diameter, 3.5- to 4-mm-long) trabeculae were obtained from the right ventricle and tied at each end with 6-0 silk thread. Each thin muscle was suspended in a 5-ml vertical constant-temperature bath that contained KH solution at 37°C. During SS contraction, the muscle was stimulated electrically via platinum bipolar electrodes with rectangular 2-ms-duration pulses at an intensity 50% above threshold delivered at 2,000-ms intervals. The trabeculae were stretched to optimal length. Right, rather than left, ventricular trabeculae were used, because it is possible to obtain a greater number of thin muscle strips from the right ventricle. The responses to Lzm-S were found to be the same between right and left, ventricular trabeculae were used, because it is possible to obtain a greater number of thin muscle strips from the right ventricle.

The cardiac autonomic FSR was determined in the RVT preparation as previously described (7, 22, 28). The pulse width of the electrical stimulus trains was increased from 2 to 20 ms, and other stimulus parameters were unchanged. After ~1 min of such pulses, or when the isometric contraction had plateaued, control responses were restored by reduction of the pulse width to 2 ms. The increase in isometric tension observed with field stimulation (FSR) was calculated as a percent increase from SS twitch amplitude: FSR = (PR – SS)/SS × 100%, where PR is the peak isometric response. In a previous study, propranolol blocked FSR in this RVT preparation (22). Measurements of FSR were obtained at ~10-min intervals. Isometric contraction measured in grams was converted to units of tension (mN/mm²), in which grams per cross-sectional area was multiplied by the acceleration due to gravity (9.80 cm/s²). Cross-sectional area was determined as follows: muscle weight/specific gravity (1.06 g/ml)/muscle length.

In the present study, two general protocols were used. The plasma concentration of Lzm-S attained in our sepsis model is ~10⁻⁷–10⁻⁶ mol/l (28). In protocol 1, a dose-response effect was determined in which this approximate range of Lzm-S concentrations was examined. Lzm-S at each concentration was added to the bath for 10 min before neural stimulation. The results obtained with Lzm-S were compared with those obtained when KH solution (50 μl) was added to the organ bath at similar intervals. In protocol 2, to ensure that the measurement of isometric contraction was stable when sequential stimulations were performed, two repetitive baseline FSR (i.e., before addition of Lzm-S or KH solution) were obtained ~10 min apart. At the third measurement interval, KH solution or Lzm-S was randomly added to an individual bath, and the results of these treatments were compared.

Lzm-S was purified from the spleens of nonseptic dogs as described in an earlier study (28) by ARVYS proteins (Stamford, CT). The turbidimetric method of Shugar (37) was used to confirm Lzm-S cleaves the high-mannose-hybrid carbohydrate structure and, therefore, was used in the present study. The ventricular trabeculae were treated with endo-F₁ for 2 h [native protein deglycosylation kit (product code N-Degly, Sigma, Oakville, ON, Canada)] to determine whether a similar inhibitory effect of Lzm-S on FSR could be observed. Two stable baseline measurements were determined, and the effect of 10⁻⁷ mol/l Lzm-S on FSR was compared between the endo-F₁-treated and nontreated groups.

To determine whether the Lzm-S-induced decline in FSR was mediated by a mechanism related to NO release, we performed initial experiments to ascertain whether the nonspecific NOS inhibitor N⁶⁻propionyl-l-arginine (L-NMMA) prevented this effect. The muscle was incubated with 10⁻⁵ mol/l L-NMMA (AG Scientific, San Diego, CA) for ~0.5 h in the organ bath in the nonstimulated condition to ensure that maximal NO inhibition could be achieved (38). FSR were obtained at consecutive Lzm-S concentrations of 10⁻⁸, 10⁻⁷, and 10⁻⁶ mol/l. These results were compared with those from appropriate control groups.

After the L-NMMA experiments, experiments were performed to determine whether relatively specific inhibitors of neuronal NOS (nNOS) or inducible NOS (iNOS) could prevent the effect of Lzm-S (36), since a specific endothelial NOS (eNOS) inhibitor is not available. The initial inhibitors were as follows: N⁶⁻propionyl-l-arginine (AG Scientific) for nNOS (21, 26) and 1400W dihydrochloride (AG Scientific) for iNOS (11). A dose-response relationship of 10⁻⁷–10⁻⁸ mol/l for these inhibitors was used to determine the predominant NOS isoform. In a final set of NOS inhibitor experiments, another nNOS inhibitor (45S-N-[4-amino-5-[(aminomethyl)[aminopentyl]-N'-nitroguanidine, TFA, 10⁻⁴ mol/l and 10⁻³ mol/l] (TFA) was also used to determine its inhibitory effect (EMD Biosciences, San Diego, CA).

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**Fig. 1.** Results from representative experiments in the 4 groups in the N⁶⁻monomethyl-l-arginine (l-NMMA) study (protocol 1). A: baseline trace for an untreated muscle. At baseline, isometric tension measured during field stimulation (FRS) was ~25% higher than steady state (SS) contraction (distance between dashed line and peak force development). Addition of lysyomse (Lzm-S, 10⁻⁷ mol/l) to the organ bath decreased SS contraction and eliminated FRS (right). B: Lzm-S caused much less decrease in FSR and SS contraction than in non-L-NMMA-treated preparation. C and D: decreases in FSR and SS contraction in Krebs-Henseleit (KH)-treated control muscles incubated with and without l-NMMA were similar to contractions in l-NMMA-incubated group to which Lzm-S was added.
After a baseline measurement of FSR, 10−6 mol/l Lzm-S decreased FRS to ~0%. In L-NMMA-treated group, Lzm-S induced only small decreases in FRS. In other KH-treated groups, decrease in FSR was similar to that in L-NMMA + Lzm-S group.

In terms of signal transduction, Lzm-S might activate receptors that are coupled to inhibitory GTP-binding proteins that are sensitive to pertussis toxin (PTX) (8, 25). On the basis of previous experiments (7), ventricular trabeculae were incubated with 1 μg/ml PTX for 2 h, and the muscle was washed. After two consecutive baseline stimulations were stable, the effect of Lzm-S on FSR was compared with the effect of KH solution on PTX-treated trabeculae.

Experiments were also performed to determine whether the decrease in FSR could be mediated by activation of soluble guanylyl cyclase and, therefore, whether the cGMP pathway might be involved in the effect of Lzm-S (20, 23). Trabecular muscles were incubated with 10−3 mol/l 1H-IH-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ; EMD Biosciences), which inhibits NO activation of soluble guanylyl cyclase (20). After a baseline measurement of FSR, 10−6−10−7 mol/l Lzm-S was added consecutively at 10-min intervals to the ODQ-treated and untreated preparations. Control groups for the effects of time and the inhibitor were also included in this protocol.

In the heart, the effects of β1- and β2-adrenoceptors (ARs) are well established and produce inotropy in ventricular myocardium. First, we determined whether the β1- and β2-adrenergic response to 10−8 mol/l isoproterenol was preserved after ventricular trabeculae were treated with 10−6 mol/l Lzm-S for ~20 min to produce a decrease in SS contraction. These results were compared with those in ventricular trabeculae treated with KH solution over an identical interval. Also, we examined the contribution of β3-AR to the Lzm-S-induced decrease in FSR (12). The pathway of β3-AR stimulation involves inhibitory G (Gi) protein signaling and results in NO release by eNOS, which resides within the domain of the caveolae (12) (see RESULTS and DISCUSSION). It was hypothesized that SR-59203A, a specific inhibitor of the β3-AR, might block the Lzm-S-induced decrease in FSR. After 30 min of incubation with 10−6 mol/l SR-59203A and two stable measurements of FSR, addition of 10−6 mol/l Lzm-S to the preparation allowed us to determine whether the decrease in FSR was prevented by SR-59203A.

Finally, we determined whether the decrease in the Lzm-S-induced decrease in FSR reflects an imbalance between neural sympathetic release of NE and neural parasymathetic stimulation. First, we assessed whether a decrease in NE release, as determined by sympathetic overflow, was the mechanism by which Lzm-S decreased FSR (35). After two baseline adrenergic stimulations were considered stable, NE overflow was measured before and after 10−6 mol/l Lzm-S was added to the preparation in which NE was measured by ELISA. The results obtained with Lzm-S alone were compared with those obtained from two other groups that were pretreated with the nNOS inhibitor TFA. In one of the two nNOS inhibitor-pretreated groups, NE overflow was measured before and after addition of 10−6 mol/l Lzm-S to the preparation; in the other nNOS inhibitor-pretreated group, NE overflow was measured before and after addition of KH solution. The results were compared among the three groups. NE was measured by NE ELISA (GenWay Biotech, San Diego, CA), which has a detection limit of 0.6 ng/ml and little cross-reactivity with other catecholamines.

In a second part of this protocol, we examined whether enhanced parasympathetic neural signaling through the muscarinic M2 receptor might also contribute to the Lzm-S-induced decrease in FSR (8, 25). In one subset of this protocol, the effect of prior treatment with the muscarinic receptor antagonist atropine (10−6−10−8 mol/l) was studied to determine whether it would prevent the effect of Lzm-S. In atropine-pretreated ventricular trabeculae, after two baseline stimulations were obtained, 10−6 mol/l Lzm-S or KH solution was administered. In a second subset of this protocol, we determined whether atropine would enhance FSR after addition of Lzm-S or KH to untreated ventricular trabeculae or ventricular trabeculae that had been pretreated with the nNOS inhibitor TFA (10−6 mol/l) or PTX. In a third subset of this protocol, after two baseline stimulations were performed, the muscarinic agonist methacholine (10−7 mol/l, acetyl-β-methacholine; Sigma) was added to determine whether this agent...
would decrease FSR in a manner similar to that described for Lzm-S. In a fourth subset of this protocol, the ventricular trabeculae were washed after addition of Lzm-S to determine whether the decrease in FSR could be restored.

Statistics. Differences among groups were determined by a two-way analysis for repeated measures of variance (between-within ANOVA) and one-way ANOVA. Student-Newman-Keuls multiple comparison test was included to determine statistical differences among treatment groups when the ANOVA was used. In the design of the experiment, of the three to four trabeculae obtained from a dog, each trabecular muscle was used for a different subset of experiments in a specific study. Values are means ± SD.

RESULTS

Two experimental protocols were performed to examine the effect of Lzm-S on FSR. In protocol 1, the dose-response effect of Lzm-S was obtained. Figure 1A shows results from the l-NMMA study. Two to three beats of SS contraction are shown at the beginning of the trace. Field stimulation was initiated at the arrow. The small dip in isometric tension at the beginning of stimulation is believed to be due to the effect of abnormal synchronization, as described by Blinks (4). This effect may be due to abnormal conduction of the action potential on initial application of field stimulation, possibly leading to an even more negative tension than with SS contraction in some instances. At baseline, field stimulation increased tension ~25%. Lzm-S (10⁻⁷ mol/l) eliminated the increase in isotropy observed with field stimulation. The mean results are shown in Fig. 2, which shows a progressive decline in FSR as higher doses of Lzm-S were added to the preparation.

In protocol 2 (Fig. 3), after two consecutive stable baseline FSR were obtained, 10⁻⁶ mol/l Lzm-S decreased the response compared with the group treated with KH solution. In the Lzm-S-treated group (n = 13), the two pretreatment baseline FSR were 18 ± 5 and 19 ± 8%, and FSR decreased to 5.6 ± 7% (P < 0.05 vs. initial value, P < 0.05 between groups, P < 0.05 vs. 2nd baseline value) after Lzm-S was added to the preparation. In the control group treated with KH solution (n = 12), the corresponding FSR were 18 ± 12, 21 ± 12, and 24 ± 15% (P < 0.05 vs. initial value).

In protocols 1 and 2, SS contraction declined progressively in the KH- and Lzm-S-treated groups. These changes occurred irrespective of the direct effect of Lzm-S on SS contraction and are shown in Tables 1 and 2 for protocols 1 and 2, respectively. The mechanism of this decline is probably related to the negative inotropic effect of excess catecholamine release, which impairs the contractile apparatus when multiple neural stimulations are performed (34), since this decline in SS was much less in time-control groups in which field stimulation was not performed (Tables 1 and 2). Moreover, Li et al. (22) showed that any decline in SS with repeated field stimulation does not affect the magnitude of FSR, which remains relatively constant over time. Additional evidence for this finding is shown in experiments described below in which atropine increased FSR, even though multiple stimulations had previously been performed (see Table 4).

Chemical removal of EE does not prevent Lzm-S-induced decrease in FSR. In a previous study, chemical removal of EE by treatment with Triton X-100 (0.5%) for 2 or 4 s eliminated the depressant effect of Lzm-S on SS contraction (27). Very short (1–5 s) exposure of the ventricular trabeculae to the detergent Triton X-100 (0.5%) has been shown to injure the endothelium but does not affect myocardial sarcoplasmic and sarcolemma membranes (3, 9). In the present study, chemical removal of EE allowed us to determine whether the Lzm-S-induced decrease in FSR could be restored. In the Lzm-S-treated group, the duration of exposure to Triton X-100 was 3 s for one muscle, 8 s for one muscle, 5 s for seven muscles, and 4 s for three muscles; in the KH-treated group, the duration of exposure to Triton X-100 was 3 s for one muscle, 8 s for one muscle, 5 s for seven muscles, and 4 s for one muscle. Because the effect of Triton X-100 on prevention of Lzm-S’s depressant effect was independent of the duration of Triton X-100 exposure, the different durations were pooled. The two baseline FSR for Triton X-100-exposed preparations of 20 ± 10 and 20 ± 10% decreased to 1.2 ± 3% with 10⁻⁷ mol/l Lzm-S (n = 13, P < 0.05 vs. baseline and P < 0.05 between groups). In the KH-treated group (n = 10), there was no change among the three conditions: 23 ± 14, 24 ± 12, and 17 ± 8%.

Table 1. Steady-state tensions in protocol 1 and in non-field-stimulated control group

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Pre-Lzm-S or KH</th>
<th>Lzm-S (10⁻⁷ mol/l) or KH</th>
<th>Lzm-S (10⁻⁶ mol/l) or KH</th>
<th>Lzm-S (10⁻⁵ mol/l) or KH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lzm-S alone</td>
<td>7</td>
<td>9.2±4.6</td>
<td>5.1±2.4†</td>
<td>4.1±2.4†</td>
<td>3.1±2.0†</td>
</tr>
<tr>
<td>l-NMMA + Lzm-S</td>
<td>9</td>
<td>9.1±2.2</td>
<td>9.0±3.1</td>
<td>7.4±2.7</td>
<td>6.5±2.8</td>
</tr>
<tr>
<td>l-NMMA + KH</td>
<td>9</td>
<td>9.0±2.9</td>
<td>9.1±3.4</td>
<td>7.9±3.1</td>
<td>6.6±3.7</td>
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<tr>
<td>KH-alone</td>
<td>13</td>
<td>8.9±3.1</td>
<td>9.1±3.7</td>
<td>8.0±3.2</td>
<td>7.1±3.7</td>
</tr>
<tr>
<td>Non-field stimulated + KH (control)</td>
<td>6</td>
<td>8.6±1.6</td>
<td>8.6±1.6</td>
<td>8.5±1.6</td>
<td>8.04±1.4</td>
</tr>
</tbody>
</table>

Values are means ± SD in mN/mm². *P < 0.05 vs. baseline 1. †P < 0.05 vs. previous condition. ‡P < 0.05 vs. other groups.

Table 2. Steady-state tensions in protocol 2 and in a non-field-stimulated control group

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Baseline 1</th>
<th>Baseline 2</th>
<th>Lzm-S (10⁻⁷ mol/l) or KH</th>
</tr>
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<tbody>
<tr>
<td>Lzm-S + field stimulated</td>
<td>13</td>
<td>7.4±2.7</td>
<td>5.9±2.1*</td>
<td>3.9±1.6†</td>
</tr>
<tr>
<td>KH + field stimulated</td>
<td>12</td>
<td>7.6±1.8</td>
<td>5.8±1.5†</td>
<td>4.4±1.9*</td>
</tr>
<tr>
<td>Non-field-stimulated + KH (control)</td>
<td>9</td>
<td>8.4±3.7</td>
<td>8.6±4.1‡</td>
<td>8.4±2.6§</td>
</tr>
</tbody>
</table>

Values are means ± SD in mN/mm². *P < 0.05 vs. baseline 1. †P < 0.05 vs. previous condition. ‡P < 0.05 vs. other groups.
Lzm-s binds to a membrane N-glycoprotein to decrease FSR. It was previously found that Lzm-S causes SS dysfunction by binding to an N-glycoprotein on EE. In particular, Lzm-S binds to the mannose β(1-4)GlcNAcβ(1-4)GlcNAc moiety of the trimannosyl core structure, where GlcNAc is N-acetylgalcosamine (18). The SS depressant effect of Lzm-S was eliminated by endo-F1, which cleaves the high-mannose-hybrid carbohydrate structures. In the present study, endo-F1 also eliminated the Lzm-S-induced decrease in FSR. In the Lzm-S-treated endo-F1 group, the two baseline measurements were 36 ± 25 and 33 ± 23%; after Lzm-S instillation, FSR remained unchanged at 43 ± 41%. In the KH-treated endo-F1 group, the two baseline measurements were 35 ± 35 and 39 ± 41%; after KH instillation, the response remained at 43 ± 46%.

NOS inhibitors prevent the decline in FSR. The nonspecific NOS inhibitor l-NNMMA (Fig. 1B) was initially used to determine whether NO production was important in the decline in FSR. As shown in Fig. 1, l-NNMMA attenuated the decrease in FSR compared with the non-l-NNMMA-treated preparation. The mean results are shown in Fig. 2. After the l-NNMMA experiments, an nNOS inhibitor and an iNOS inhibitor were used to determine whether one of these isoforms could be excluded. The initial nNOS inhibitor (i.e., Nω-propyl-l-arginine) is a potent and selective inhibitor of nNOS relative to iNOS (3,158-fold) and eNOS (149-fold), whereas the iNOS inhibitor (i.e., 1400W dihydrochloride) is highly selective for this isoform (1,2a,11,21,26). Because the iNOS inhibitor at 10⁻³–10⁻⁵ mol/l had little preventive effect on field stimulation (i.e., FSR remained at 5%), further dilutions of this inhibitor were not performed. On the other hand, the nNOS inhibitor at 10⁻²–10⁻⁸ mol/l maintained FSR at ~15% [values were only slightly lower (P < 0.05) than the baseline value of 25 ± 9%]. FSR decreased to 10.3 ± 5% at 10⁻⁹ mol/l and to 0 at 10⁻¹⁰ mol/l.

In other experiments, the nNOS inhibitor TFA, which has much greater specificity for nNOS than for eNOS, also prevented Lzm-S-induced dysfunction (Table 3).

Lzm-S mediates a decrease in FSR by Gi protein signaling and activation of the cGMP pathway. In terms of signal transduction, the results show that a Gi protein was involved in the Lzm-S-induced reduction of FSR. As shown in Fig. 4, PTX prevented the depressant effect of Lzm-S. In addition, activation of the cGMP pathway was also involved in the Lzm-S-induced decline in FSR. To assess involvement of the cGMP pathway in the decline of FRS, we determined whether incubation of the muscles with the guanylyl cyclase inhibitor ODQ prevented this decline. As shown in Fig. 5, ODQ markedly inhibited the Lzm-S-induced reduction in FSR.

Table 3. Effect of TFA on Lzm-S-induced reduction in FSR

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Pretreatment</th>
<th>Baseline 1</th>
<th>Baseline 2</th>
<th>Lzm-S (10⁻⁶ mol/l) or KH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lzm-S only</td>
<td>12</td>
<td></td>
<td>20 ± 9</td>
<td>21 ± 8</td>
<td>7 ± 8%†</td>
</tr>
<tr>
<td>TFA (10⁻⁴ mol/l) + Lzm-S</td>
<td>6</td>
<td></td>
<td>16 ± 6</td>
<td>15 ± 5</td>
<td>12 ± 3</td>
</tr>
<tr>
<td>TFA (10⁻⁶ mol/l) + Lzm-S</td>
<td>8</td>
<td></td>
<td>16 ± 4</td>
<td>21 ± 10</td>
<td>30 ± 15</td>
</tr>
<tr>
<td>TFA (10⁻⁴ mol/l) + KH</td>
<td>6</td>
<td></td>
<td>21 ± 6</td>
<td>20 ± 5</td>
<td>21 ± 9</td>
</tr>
<tr>
<td>TFA (10⁻⁶ mol/l) + KH</td>
<td>8</td>
<td></td>
<td>21 ± 5</td>
<td>23 ± 7</td>
<td>22 ± 8</td>
</tr>
<tr>
<td>KH only (time control)</td>
<td>12</td>
<td></td>
<td>21 ± 15</td>
<td>23 ± 12</td>
<td>26 ± 12</td>
</tr>
</tbody>
</table>

Values are means ± SD in percent. TFA, (4S)-N-[4-amino-5-(aminoethyl)aminopentyl]N'-nitroguanidine; TFA; FSR, response to field stimulation. *P < 0.05 vs. baseline. †P < 0.05 vs. other groups.

Lzm-s does not affect β₁- and β₂-AR function. Although Lzm-S decreased FSR, the response to isoproterenol was preserved. In these experiments, Lzm-S decreased SS contraction to 65 ± 11% of the pre-Lzm-S value; nevertheless, isoproterenol increased isometric contraction 59 ± 53% in the Lzm-S-treated group and 43 ± 24% in the KH-treated group (Fig. 6). Furthermore, in ventricular trabeculae treated with 10⁻⁶ mol/l SR-59203A for 30 min, there was no evidence that β₁-AR blockade prevented the Lzm-S-induced reduction of FSR. The two pre-Lzm-S measurements of 18.5 ± 9 and 20 ± 9% decreased to 1.1 ± 9% when 10⁻⁶ mol/l Lzm-S was added to the preparation (n = 5, P < 0.05 vs. baseline).

Lzm-S did not change the sympathetic release of NE. There were no differences in the increases in NE concentrations, expressed as absolute value or percentage, among the three groups. Because the prestimulation concentrations were sometimes small, large percent increases after stimulation that were not reflected by the changes in the absolute values were often noted. In the group treated with Lzm-S alone, the increase before vs. after stimulation was 39 ± 40 vs. 48 ± 60 mg/ml (mean percent increase = 414 ± 1,071%); in the group treated with the NOS inhibitor + Lzm-S, the increase before vs. after stimulation was 26 ± 10 vs. 35 ± 5 mg/ml (mean percent increase = 135 ± 202%); in the group treated with the NOS inhibitor + KH, the increase before vs. after stimulation was 38 ± 43 vs. 46 ± 55 mg/ml (mean percent increase 118 ± 57%).
The parasympatholytic agent atropine inhibits the Lzm-S-induced decrease in FSR. Enhanced parasympathetic neural signaling through the muscarinic M2 receptor might also contribute to the Lzm-S-induced decrease in FSR (8, 25). In response to atropine pretreatment, the Lzm-S-induced reduction of FSR was eliminated (Fig. 7). Pretreatment with atropine blocked the effect of 10^{-4}–10^{-7} mol/l Lzm-S, but the effect of Lzm-S was not blocked at 10^{-8} mol/l (Fig. 8). There was no effect of atropine pretreatment in the KH-treated group when multiple stimulations were performed. In other experiments, we administered atropine after Lzm-S to determine whether the depressant effect of Lzm-S could be reversed. The results are shown in Table 4. In general, atropine pretreatment increased FSR in all the KH- and Lzm-S-treated groups, and this effect was particularly evident after PTX incubation. Moreover, a higher dose of atropine (4 × 10^{-5} mol/l) increased the mean FSR in the KH-treated nonincubated group (n = 4) from 42 ± 50 to 238 ± 119% (see DISCUSSION). Experiments were also performed to determine whether the parasympathetic agent methacholine (10^{-5} mol/l) would mimic the depressant effect of Lzm-S. In this experiment (n = 5), the two baseline FSR of 22 ± 12 and 20 ± 9% decreased to 0 ± 0% after methacholine treatment (Fig. 9).

Finally, to determine whether the depressant effect of Lzm-S was reversible, we washed the trabecular preparation three times with KH solution. Tight binding of Lzm-S to its receptor could account for the more modest atropine response in the nonincubated Lzm-S-treated group in Table 4. In the trabecular preparation washed with KH solution, the inotropic FSR in the Lzm-S-treated group was only partially restored. In the group treated with 10^{-6} mol/l Lzm-S, the two FSR baseline values were 23 ± 18 and 22 ± 19%; addition of Lzm-S decreased FSR to 4 ± 12% (n = 8, P < 0.05 vs. baseline). Washing only partially increased the response to 14 ± 22% (P < 0.05 vs. post-Lzm-S). In the KH-treated control group, the two FSR baseline values were 31 ± 21 and 22 ± 12%; addition of KH solution decreased FSR to 19 ± 9% (n = 6). After the preparation was washed, FSR remained at 17 ± 14%.

**DISCUSSION**

In the present study, we have shown that the pathway by which Lzm-S reduces FSR is different from the pathway by which Lzm-S reduces SS isometric contraction. The mecha-
Lysosome impairs cardiac adrenergic response in sepsis

Fig. 9. Results from a representative experiment in which methacholine was administered after 2 stable stimulations. The first few beats represent SS contractions, the first 2 of which are shown at higher speed. Arrow indicates stimulation. Methacholine decreased FSR in a manner similar to that described for Lzm-S.

Table 4. Effect of atropine on FSR

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Pretreatment</th>
<th></th>
<th></th>
<th>Post-Lzm-S (10⁻⁶ mol/l)</th>
<th>Post-Atropine (10⁻⁶ mol/l)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Baseline 1</td>
<td>Baseline 2</td>
<td></td>
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</tr>
<tr>
<td>No incubation</td>
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<td>17±6</td>
<td>18±6</td>
<td></td>
<td>5±5</td>
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<tr>
<td>TFA (10⁻⁶ mol/l)</td>
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<td>17±6</td>
<td>15±5</td>
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<td>47±40*</td>
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<td>PTX (1 µg/ml)</td>
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<td>27±9</td>
<td>25±10</td>
<td></td>
<td>24±3</td>
<td>62±24*</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Post-Lzm-S (10⁻⁶ mol/l)</td>
<td>Post-Atropine (10⁻⁶ mol/l)</td>
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</tr>
<tr>
<td>No incubation</td>
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<td></td>
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<td>41±30</td>
<td></td>
<td>32±24</td>
<td>87±109†</td>
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Values are means ± SD in percent. PTX, pertussis toxin. *P < 0.05 vs. previous condition. †P < 0.05 vs. other groups.

The mechanism of Lzm-S-induced SS depression is initiated by Lzm-S binding to EE (27), which results in NO generation. NO then diffuses from EE to adjacent myocytes and, by activation of the NO-cGMP pathway, leads to myocardial depression. However, chemical removal of EE by exposure of ventricular trabeculae to Triton X-100 for up to 8 s did not eliminate the Lzm-S-induced adrenergic dysfunction, even though 2 s of exposure nearly eliminated the Lzm-S-induced decrease in SS contraction. This occurred even though identity of signal pathways for SS depression and FSR decrease was our primary hypothesis and the methodological approach was identical to that used in our previous study. Since >8 s of exposure to Triton X-100 might affect membranes other than those of the endothelium (3, 9), we believed that it was unlikely that EE was involved in the Lzm-S-induced decrease in FSR.

Although EE did not contribute to the Lzm-S-induced reduction of FSR, we found that the mechanism was attributable to NO release, since we were able to block this reduction by multiple NOS inhibitors. The nNOS inhibitors showed a significant beneficial effect, whereas the iNOS inhibitor improved the adrenergic response only marginally. Since the contribution of the eNOS isoform could not be directly tested for the lack of a specific inhibitor, the nNOS and/or eNOS isoform was most likely involved. Whereas the prevalent location of the nNOS isoform would be the sympathetic and parasympathetic nerves, eNOS and nNOS are found in the myocardium (8, 25). We also showed that NO release during neural stimulation results in activation of the cGMP pathway, since ODQ, an inhibitor of this activation, restored FSR. These results indicate that NO-cGMP signaling is involved in the pathway by which Lzm-S decreases the inotropic FSR.

We also investigated whether Lzm-S might mediate the decrease in FSR by impairment of β₁- and β₂-AR; therefore, we administered isoproterenol to determine whether this β-adrenergic pathway was intact. In the Lzm-S-treated group, the relative increase in isometric contraction to isoproterenol was similar to that of the KH-treated group. In addition, we tested whether Lzm-S might activate the β₃-AR to cause adrenergic dysfunction, since it acts via NO-cGMP signaling (12). Receptor blockade of the β₃-AR by SR-59203A did not reverse the Lzm-S-induced decrease in FSR. We also considered that Lzm-S might inhibit NE release from the sympathetic nerves, but we found that NE release was not different among the groups examined. Thus another pathway was involved in the effect of Lzm-S.

In terms of this potential pathway, the Lzm-S-induced decline in FSR could be attenuated by PTX treatment, and thus a heterotrimer Gi protein was involved in the Lzm-S-induced reduction of FSR. Gi proteins may be found at the sympathetic presynaptic site and on the myocyte in relation to β₂-AR; therefore, SR-59203A did not inhibit the depressant activity of Lzm-S, an effect that was not observed in the present study. In addition, stimulation of the β₃-AR also involves a Gi protein pathway (12) (Fig. 10). However, since blockade of this receptor by SR-59203A did not inhibit the depressant activity of Lzm-S, we do not think that this mechanism was involved.

Nevertheless, it is the interplay between the sympathetic and parasympathetic systems that determines the ultimate FSR (8, 25). In response to parasympathetic stimulation, acetylcholine diffuses across the synaptic cleft to bind to the myocyte muscarinic (M₂) receptor, which causes the release of NO to diminish FSR, although it is debated whether myocyte eNOS or nNOS is the isoform involved (8, 25) (Fig. 10). In the myocyte, NO may reside in microdomains associated with the caveolae, among other locations. Within caveolae, NO may inhibit calcium influx by cGMP-dependent inhibition of the L-type calcium channel. Relatively increased activation of the
FSR increased to a degree equal to or only marginally less than that in the KH-treated groups. Any differences between the KH- and Lzm-S-treated groups were small and, to some extent, may be attributable to Lzm-S’s tight binding to the membrane N-glycoprotein as per the washing experiments.

We further showed that the parasympathomimetic agent methacholine would decrease FSR. Methacholine also markedly decreased SS to an extent similar to that induced by Lzm-S. In addition to activation of muscarinic receptors, parasympathetic agents are known to cause NO release in EE (24), so that a decrease in SS secondary to this release of NO would be anticipated in our preparation. Thus, during field stimulation, the relative effects of NE release and parasympathetic stimulation compete to determine FSR. The net effect favors the sympathetic component, leading to an increase in contractility representing the adrenergic response. Lzm-S increases the parasympathetic component and, therefore, diminishes the adrenergic response. The action of Lzm-S can be blocked by atropine.

Furthermore, as shown in Table 4, atropine enhances FSR, particularly under conditions of NOS inhibition and PTX incubation. We explain this effect as follows. Although a high dose (10^−4 mol/l) of atropine could block inhibition of FSR by acetylcholine, it is evident that acetylcholine blockade was not complete, since when an even higher concentration (4 × 10^−4 mol/l) of atropine was administered to the nonincubated KH-treated group, FSR increased further to 185% compared with 42%. FSR was therefore greater under conditions of NOS inhibition and PTX incubation, since for any acetylcholine release, there was less Gi protein signaling and myocyte NOS activation to diminish the inotropic FSR in these groups.

Nevertheless, our results do not show the precise mechanism by which Lzm-S modulated the parasympathetic pathway to decrease FSR. Lzm-S could have directly stimulated the myocyte M2 receptor or enhanced its sensitivity to acetylcholine. Alternatively, Herring and Paterson (17) showed that the NO-cGMP pathway facilitates parasympathetic neuronal release of acetylcholine during vagal stimulation. Lzm-S may have acted to stimulate a receptor located on the parasympathetic neuron to activate nNOS, an effect that would have resulted in a decrease FSR. Lzm-S, a mediator of sepsis, inhibits the inotropic FSR and that effect can be blocked by atropine.

In terms of our preparation, multiple field stimulations produced a decrease in SS contraction that was out of proportion to the response to no stimulation (Tables 1 and 2). Furthermore, the decline in SS contraction appeared to be greater in Lzm-S-treated ventricular trabeculae, a finding consistent with the additional effect of Lzm-S on NO-induced myocardial depression (Tables 1 and 2). Although the mechanism by which multiple field stimulations accelerate the decline of SS is not clear and may be multifactorial, it is important to note that FSR remains little changed when repeated stimulations are performed in control, untreated ventricular trabeculae. We also showed this effect in an earlier study when an identical preparation was used and where FSR was examined in some detail (22). In the present study, we
always used concomitant control (i.e., KH-treated) groups to account for the effects of multiple stimulations and time. In protocols 1 and 2, FSR was decreased in the Lzm-S-treated group compared with the KH-treated group. In addition, atropine administration produced a relative and often absolute increase in FSR but only a slight decrease in SS contraction. Thus the Lzm-S-induced decrease in FSR does not appear to be due to deterioration of the preparation, since FSR could be increased by atropine.

Lzm-S is a newly discovered mediator of myocardial depression in septic shock (28). The in vivo contribution of Lzm-S to cardiovascular collapse in this condition was previously demonstrated in our E. coli sepsis model, in which chitotriose (\(N,N',N''\)-triacetylchitotriose), an inhibitor of Lzm-S, prevented the fall in mean arterial blood pressure, cardiac output, and stroke work otherwise observed (29). We also found that chitotriose could inhibit the Lzm-S-induced decrease in FSR in the RVT preparation (28). Lzm-S mediates SS depression and the decrease in FSR by a signaling mechanism that involves NO release (27). The release of NO by EE causes a decrease in SS contraction, although the receptor for this release is not known. On the other hand, during neural stimulation, an increase in NO via parasympathetic signaling inhibits FSR. These findings would be consistent with the compartmentalization that is characteristic of NO (8, 25). Although NO would derive from different sources, both effects would contribute to the cardiovascular collapse in septic shock.

In clinical medicine, nonspecific NOS inhibitors have not been shown to be beneficial in the treatment of septic shock (14). This negative effect appears to be related to the fact that some NO production is required for maintenance of regional organ blood flow. The beneficial aspect of Lzm-S inhibitors is that they would inhibit NO production only in localized regions to which Lzm-S binds. Such a localized effect would not shut off total body NO production and would possibly lead to more beneficial clinical results. Burgess et al. (6) showed that plasma Lzm-S concentration was increased in humans who presented with abdominal sepsis. Thus we believe that the Lzm-S-induced declines of SS contraction and FSR in this canine model are also applicable to the cardiovascular depression found in clinical medicine.

The novel finding of this study is that it identifies an inflammatory mediator released in septic shock that may inhibit the cardiac neural adrenergic response. The mechanism seems to relate to relatively enhanced stimulation of the parasympathetic system that can be blocked by atropine. On the basis of our in vivo and in vitro experiments (18, 27–29), we therefore suggest that inhibitors of Lzm-S, by improving SS contraction and the neural adrenergic response, offer a potential strategy for the reversal of cardiovascular collapse in septic shock.

GRANTS

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REFERENCES


