Vesnarinone Restores Contractility and Calcium Handling in Early Endotoxemia

Koh Takeuchi, MD; Pedro J. del Nido, MD; Dimitrios N. Poutias, BS; Douglas B. Cowan, PhD; Mamoru Munakata, MD; Francis X. McGowan, Jr, MD

Background—Endotoxin (lipopolysaccharide, LPS) is a trigger of the systemic inflammatory response. We have previously found that vesnarinone and amrinone, when given before LPS, prevented cytokine production and LPS-related cardiac dysfunction. We tested the hypothesis that vesnarinone would improve intracellular Ca\(^{2+}\) handling and calcium-activated contractile force after the onset of endotoxemia.

Methods and Results—Adult rabbits received a bolus injection of LPS or vehicle. Vesnarinone (3 mg/kg) was given intravenously 90 minutes later. Two hours after LPS administration, hearts were perfused in the isolated Langendorff mode. Peak left ventricular developed pressure, ±dp/dt, oxygen consumption (MV\(\dot{O_2}\)), and rate\(\times\)pressure product were evaluated in conjunction with fluorescent spectroscopic determinations of intracellular calcium concentrations (Ca\(_i\)) and the rate of Ca\(_i\) transient decline during diastole (\(t_{Ca}\)). Peak left ventricular developed pressure and ±dp/dt were significantly lower in the LPS group. These were completely restored by vesnarinone. There was significantly slower diastolic calcium removal (increased \(t_{Ca}\)) in LPS hearts that was also corrected by vesnarinone; however, the cytosolic calcium overload characteristic of LPS hearts was only partially improved. Reduced mechanical inefficiency (the ratio of rate-pressure product to MV\(\dot{O_2}\)) and myofilament sensitivity to Ca\(_i\) were also significantly improved by vesnarinone.

Conclusions—Acute endotoxemia caused contractile protein calcium insensitivity, oxygen wastage, and abnormal calcium cycling. Vesnarinone, given in the rescue mode, normalized LPS-induced myocardial dysfunction and partially restored abnormal calcium cycling. Although the mechanisms responsible for these effects require further clarification, it appears that agents such as vesnarinone may be useful to treat inflammatory-induced myocardial dysfunction. (Circulation. 2000;102[suppl III]:III-365-III-369.)

Key Words: calcium ■ contractility ■ proteins ■ inflammation

Endotoxin (lipopolysaccharide, LPS) is a prime trigger of the systemic inflammatory response that produces multiple organ dysfunction during sepsis.\(^1\) LPS release during cardiopulmonary bypass has also been linked to cardiac dysfunction.\(^2\) In experimental endotoxemia models, systolic dysfunction and reduced contractile reserve can be observed within 1 to 4 hours; these occur in the absence of systemic acidosis, hypotension, or decreased coronary perfusion, and before significant increases in circulating proinflammatory cytokines.\(^3,5\) More recently, we have shown that myocardial intracellular calcium (Ca\(^{2+}\)) handling and contractile protein sensitivity to Ca\(^{2+}\)i are rapidly impaired (within 2 hours) by endotoxemia.\(^6\) These rapid effects of endotoxin on myocardial function and calcium cycling were not improved by \(\beta\)-agonist administration. We have also found that vesnarinone, which has phosphodiesterase-inhibiting, ion channel, and immunomodulating activity, significantly reduced cytokine production and myocardial contractile dysfunction when given before LPS administration.\(^7\) The purpose of the present study was to test the hypothesis that vesnarinone would improve intracellular Ca\(^{2+}\) handling and calcium-activated contractile force after the onset of LPS-induced contractile dysfunction.

Methods

Reagents

Vesnarinone was a gift from Otsuka Pharmaceutical Co. All other chemicals were purchased from Sigma Chemical Company, unless otherwise noted, and were of the highest grade available. LPS (lot 126H4099) was also purchased from Sigma. The calcium-sensitive, cell-permeable fluorescent dye Rhod-2AM was from Molecular Probes.

Animal Model

Animal procedures received institutional approval and were conducted in conformity with the Guiding Principles in the Care and Use of Animals of the American Physiology Society and the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH publication No. 85-23, revised, 1985). New Zealand White rabbits (2.5 to 3.0 kg) received a bolus injection of 0.2 mg/kg Salmonella typhimurium LPS dissolved in 1 mL/kg sterile saline (LPS group, n=7) or sterile saline alone (1 mL/kg, control.
group, n=6). Ninety minutes after LPS administration or saline administration (n=6 each), animals received 3 mg/kg vesnarinone as an intravenous infusion. Two hours after LPS or saline administration, animals received ketamine (100 mg/kg) and heparin (1000 U) intravenously. Their hearts were then rapidly excised, placed in ice-cold Krebs-Henseleit (K-H) buffer, and rapidly perfused retrograde through the aorta in the Langendorff mode with the use of modified K-H crystalloid perfusate, exactly as described previously. A latex fluid-filled balloon connected to a micromanometry catheter (Miller Instruments) was used for isovolumic left ventricular function measurements. Hearts were paced at 150 bpm by atrioventricular pacing wires sutured to the right atrium and the free wall of the right ventricle. Peak developed left ventricular (LV) pressures were measured at an LV end-diastolic pressure of 5 mm Hg. Coronary flow was measured by timed collection of the coronary effluent. Oxygen content was calculated from the measured oxygen tension (ABL-3 Acid-Base Laboratory, Radiometer) of simultaneous aortic perfusate and coronary venous effluent samples; myocardial oxygen consumption (MV̇O₂) was calculated as the measured arteriovenous oxygen difference multiplied by coronary flow and divided by dry heart weight. Relative mechanical efficiency was determined by the ratio of work, quantified by the rate×pressure product (RPP), to myocardial oxygen consumption (RPP/MV̇O₂).

## Calcium Measurements
Measurements of beat-to-beat intracellular calcium transients was performed as we have previously described and validated in detail. Because Rhod-2 has no spectral shift when it binds calcium, it is necessary to account for changes in tissue Rhod-2 concentration over time (eg, leakage or photobleaching) as well as differences in dye loading. To do this, F₅₈⁹ was corrected for tissue absorbance as follows. Excitation light was scanned at wavelengths between 524 nm (peak Rhod-2 absorbance in cardiac tissue) and 589 nm (reference wavelength at which there is insignificant Rhod-2 absorbance) and the ratio of reflected light at 524:589 used to estimate tissue dye concentration. The calcium fluorescence signal was also corrected for tissue autofluorescence by subtracting the fluorescence spectra of the heart before Rhod-2 loading from that of the Rhod-2–loaded heart. Instantaneous ratioing of the fluorescent intensity (F) at 589 nm to reflected excitation light at 524 nm was done electronically to correct for heart motion. Calcium transient values were expressed as peak fluorescent intensity corrected for autofluorescence divided by the absorbance correction for dye concentration (F/A).

After stabilization of dye loading and baseline signal acquisition, beating hearts were perfused in a stepwise fashion for 2 minutes each with K-H buffer containing 0.75, 1.25, and 2.25 mmol/L calcium. In this way, the rate of extracellular calcium (Caₑ) to intracellular calcium (Caᵢ) and Caᵢ to contractile force could be demonstrated. LV pressure and Rhod-2 fluorescence data were recorded simultaneously. All data analysis was performed with commercially available software (Sigma Plot, Jandel Scientific). The rate of the Caᵢ transient decline during diastole, τCa was assessed by curve-fitting to a monoexponential function as described previously, using the following equation:

\[ \text{Ca}_i(t) = (\text{Ca}_\text{inf} - \text{Ca}_\text{init}) \times \exp(-t/\tau) + \text{Ca}_\text{init} \]

where \( \text{Ca}_\text{init} \) is \( \text{Ca}_\text{inf} \) at minimum d[Ca]/dt and \( \text{Ca}_\text{init} \) is \( \text{Ca}_\text{inf} \) extrapolated to infinite time. τ was calculated with this equation between 20% and 70% decline of transient amplitude.

## Statistical Analysis
All values are reported as mean±SEM. Data are from 6 experiments each. Multiple group comparisons were made using ANOVA followed by the Bonferroni procedure. Differences were considered significant at \( P<0.05 \) with adjustment made for multiple comparisons.

## Results

### Effects of LPS and Vesnarinone

One of 7 animals that received LPS alone died (at ≈90 minutes after LPS administration); there were no deaths in any of the other groups. No animal, regardless of treatment group, was hypotensive (mean arterial pressure was ≥50 mm Hg in all animals) before cardiac harvesting. Peak developed LV pressure was significantly lower in LPS hearts than in normal hearts (91±2 versus 110±5 mm Hg, \( P=0.05 \)). This was restored by vesnarinone administration to a supranormal level (138±8 mm Hg, \( P=0.01 \) versus LPS alone) that was not significantly different from the response of normal hearts to vesnarinone (142±5 mm Hg). Positive dp/dt (\( + \text{dp/dt} \)) and negative dp/dt (\( - \text{dp/dt} \)) were also significantly lower in LPS hearts than in control hearts; these parameters were also restored by vesnarinone treatment (Figure 1).

Typical Rhod-2 fluorescence tracings are shown in Figure 2. Both systolic and diastolic calcium concentrations were significantly higher in LPS-treated hearts than in normal hearts. The rate of diastolic calcium removal (\( \tau \text{Ca} \)) was significantly lower in LPS hearts than in normal hearts. Although vesnarinone corrected \( \tau \text{Ca} \) (Figure 3), it only incompletely restored cytosolic calcium levels toward normal (Figure 4). As can be seen in Figure 4, LPS hearts developed significantly less pressure despite higher cytosolic calcium concentrations; vesnarinone treatment of LPS hearts was associated with significantly increased contractile force, but...
Figure 3. Effects of LPS, vesnarinone (Ves), or LPS plus vesnarinone on rate of diastolic calcium removal (tCa) 2 hours after endotoxin administration. Data are mean±SEM, n=6 each.

with only a modest reduction in Ca. This apparent improvement in myofibrillar calcium sensitivity (increased force at the same or somewhat lower calcium concentration) was also seen in control hearts exposed to vesnarinone (Figure 4).

Response to Calcium-Induced Inotropy
The ability to increase contractility in response to increasing extracellular calcium (calcium-induced inotropy) from 0.75 mmol/L to 2.25 mmol/L was assessed. The ratio of increase in pressure to the increase in intracellular calcium produced by increasing perfusate calcium was (arbitrary units) 11.64 in control hearts, 16.0.5 in LPS hearts (P<0.01 versus LPS) and 6.62 in vesnarinone-treated LPS hearts (P<0.05 versus LPS alone and control). In other words, contractility in normal hearts was very responsive to the small changes in Ca produced by increasing extracellular calcium. In contrast, increasing perfusate calcium in LPS hearts led to much larger changes in Ca, which were nevertheless accompanied by minimal increases in contractility. Although contractile force was normalized in LPS-vesnarinone hearts, there was still increased calcium cycling (see Figure 4) leading to a lower value for the efficiency of calcium-induced inotropy.

Overall mechanical efficiency was estimated from the ratio of the LV rate-pressure product to oxygen consumption (RPP/MV O 2) in response to calcium-induced inotropy. As can be seen in Figure 5, LPS hearts developed significantly less force despite higher oxygen consumption, and this effect was corrected by vesnarinone.

Discussion
The major finding of this study was that vesnarinone, given in the “rescue mode” 90 minutes after the onset of endotoxemia, improved LPS-stimulated abnormalities in myocardial function. Our data do not allow us to precisely define the mechanism(s) of action of vesnarinone in this setting. However, it is clear that vesnarinone led to substantial improvements in contractility, contractile responsiveness to calcium, diastolic calcium removal, and mechanical efficiency.

One or more intracellular sites may be responsible for the observed calcium handling abnormalities during endotoxemia. In the adult rabbit (as in the adult human) heart, cytosolic calcium is primarily (~75% of calcium influx) determined by the sarcoplasmic reticulum (SR). The rate of [Ca2+] transient decline is thus mainly a function of the SR Ca2+-ATPase that transports calcium from the cytosol into SR; other, quantitatively smaller sites include the sarcolemmal Na+/Ca2+ exchanger that transports Ca2+ from the cytosol into the extracellular space and buffering of Ca2+ by intracellular proteins. We therefore speculate that vesnarinone improved SR Ca-ATPase function as part of its effect to speed diastolic calcium removal. Increased production of cAMP increases SR calcium uptake by stimulation of the SR Ca-ATPase and enhances diastolic relaxation and increases inotropic state; thus, phosphodiesterase inhibition may explain several of the observed effects of vesnarinone. However, in a similar model, dobutamine did not improve contractility or calcium handling when given 2 hours after LPS despite evidence of preserved β-adrenergic signaling. It is therefore unlikely that phosphodiesterase-mediated increases in cAMP are entirely responsible for the effects observed in the present study. It is also interesting that vesnarinone did not completely normalize cytosolic calcium levels despite correcting the rate of diastolic calcium removal. After vesnarinone, LPS hearts still appeared “leaky” to calcium,
as demonstrated by higher calcium levels and greater increases in $C_a$, when exposed to higher perfusate calcium. Whether this was due to decreased control of transmembrane calcium flux or to abnormal opening of the SR calcium release channel, as may be produced by oxygen and nitrogen radical species, requires further study. 10

There are several advantages to the use of Rhod-2 to measure intracellular calcium transients. It excites in the visible range and thus tissue absorbance, autofluorescence, interference from other endogenous fluorophores, and photo-bleaching are low. It efficiently loads into the cell at 37°C with minimal uptake into nonmyocytes or intracellular organelles, displays high quantum yield on binding calcium, and has a favorable $K_b$ for calcium relative to the contractile proteins (710 nmol/L in the presence of 0.5 mmol/L myoglobin). 8 Overall, these properties give good specificity, depth of tissue penetration, large dynamic range, and detailed transient morphology. However, one disadvantage is that Rhod-2 does not exhibit a shift in excitation or emission wavelength when binding calcium; one must measure and correct for changes in dye concentration (see Methods section). We also corrected for motion artifact by gently constraining the heart against an optical window and using a reflected light reference signal correction; thus, motion artifact accounts for <2% to 3% of signal.

The cause of reduced myofibrillar calcium sensitivity in LPS hearts, particularly after only 2 hours of exposure, is uncertain. The number of potential mediators released by LPS is large and includes tumor necrosis factor-α, interleukin-1β, platelet activating factor, endothelin, and nitric oxide. 1–5, 11–14 These can stimulate numerous intracellular signaling pathways, including phospholipases, protein kinase C, and sphingomyelinase, which can then modify calcium regulatory sites in the contractile proteins as well as in the sarcolemma, sarcoplasmic reticulum, and mitochondria. Contractile protein sensitivity to calcium may be directly affected by phosphorylation of sites that regulate calcium binding (e.g., troponin I) by production of cGMP, activation of protein kinase C, and so forth. Metabolic factors such as ATP, pH, Mg$^2+$, $P_i$, and the free energy of ATP hydrolysis are also known to alter contractile protein calcium sensitivity. Results from a previous study suggest that LPS increased $P_i$ and decreased contractile calcium sensitivity. 6, 11, 15, 16

We speculate that the primary effect of vesnarinone is through alterations of one or more of the aforementioned signaling pathways. Additional pharmacological effects of vesnarinone include sodium channel opening, decreased inward and outward potassium currents, and prolonged action potential duration. It has been shown to have various “immunomodulatory” effects that include inhibition of cytokine production and viral replication. We have previously demonstrated that vesnarinone and amrinone prevented or reduced many features of the acute endotoxic response, such as LPS-induced death, fever, acidosis, cardiac dysfunction, and elevated plasma cytokine concentrations. 7 More recently, we found that these compounds prevented initiation of inflammatory signaling by preventing activation of NFκB, a transcription factor that leads to expression of a wide array of cytokine and other stress genes. 17 The mechanisms of these effects are uncertain but may in part be due to increased cAMP production, leading to altered gene expression and to inhibition of “stress-induced” activation of specific membrane receptors and the signaling pathways linked to them, including those leading to the production of phosphatidic acid and ceramide. 7, 18–22 An ability to specifically inhibit stress or cellular activation signals may be a potential advantage compared with other agents with anti-inflammatory properties such as corticosteroids.

We conclude that relatively brief exposure (2 hours) to LPS in vivo caused contractile dysfunction, abnormal calcium cycling, and oxygen wastage. Similar abnormalities of calcium handling and contractile protein calcium sensitivity have been found in failing hearts and after ischemia-reperfusion. 15, 23–25 The ability to completely restore contractile function and mechanical efficiency, improve abnormal calcium cycling, and inhibit inflammatory signaling suggests that agents such as vesnarinone may have a role in the treatment of myocardial dysfunction in settings such as cardiopulmonary bypass, sepsis, transplant rejection, and myocarditis.

Acknowledgments

This work was supported in part by National Institutes of Health grants HL-52589 (to Dr McGowan) and HL-42607 (to Dr del Nido).

References


Vesnarinone Restores Contractility and Calcium Handling in Early Endotoxemia
Koh Takeuchi, Pedro J. del Nido, Dimitrios N. Poutias, Douglas B. Cowan, Mamoru Munakata
and Francis X. McGowan, Jr

Circulation. 2000;102:Iii-365-Iii-369
doi: 10.1161/01.CIR.102.suppl_3.III-365
Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2000 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/102/suppl_3/Iii-365

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation is online at:
http://circ.ahajournals.org/subscriptions/