Angiotensin II exacerbates lipopolysaccharide-induced contractile depression in rabbit cardiac myocytes

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Yasuda, Satoshi, and Wilbur Y. W. Lew. Angiotensin II exacerbates lipopolysaccharide-induced contractile depression in rabbit cardiac myocytes. Am. J. Physiol. 276 (Heart Circ. Physiol. 45): H1442–H1449, 1999.—In sepsis, lipopolysaccharide (LPS) depresses cardiac function by inducing production of nitric oxide (NO) and its second messenger cGMP. LPS also stimulates Ang II production. We hypothesized that Ang II modulates the cardiac response to LPS in adult rabbit cardiac myocytes incubated with LPS (10 ng/ml) and Ang II (100 nm) alone or in combination. LPS induced contractile depression and increased cardiac cGMP, both of which were exacerbated by Ang II. 

METHODS

LPS (10 ng/ml) or Ang II (100 nm) was added to cultured adult rabbit cardiac myocytes for 6 h. All experiments were conducted in accordance with institutional guidelines and the Guide for the Care and the Use of Laboratory Animals (Department of Health and Human Services, Publication No. (NIH) 85–23, Revised 1985). LPS is a lipopolysaccharide (LPS), the outer membrane glycolipid of gram-negative bacteria, which releases a cascade of endogenous mediators that cause hypotension, multiorgan failure, and septic shock (3, 27). LPS induces iNOS (21) and increases NO-cGMP levels, which are associated with depressed cell shortening with no alterations in Ca2+ transients ( indo-1 fluorescence), indicating a decreased myocardial response to Ca2+. Ang II (100 nm) alone had no effect. However, Ang II with LPS produced higher cGMP levels (1.025 ± 113 fmol/mg protein, n = 16, P < 0.05 vs. LPS alone), impaired cardiac contractility, decreased Ca2+ handling, and increased mitochondrial activity (MTS assay). The interaction between Ang II and LPS is synergistic on the activation of NO-cGMP pathways to induce dose-dependent impairments in excitation-contraction coupling in cardiac myocytes.

The cardiac effects of LPS are modulated by several systems (β-adrenergic and endothelins) that are activated to counteract hypotension and cardiac depression during sepsis. LPS-induced contractile depression is enhanced by β-adrenergic activation (39). Endothelin-1, a potent vasoconstrictor that increases during sepsis, attenuates NO-cGMP responses (through ETA receptors) to ameliorate the severity of LPS-induced contractile depression (38). Another major system activated during sepsis is the renin-angiotensin system, causing a rise in circulating renin and Ang II levels (9, 32). LPS also activates Ang II in local tissues. Activation of the cardiac renin-angiotensin system has important paracrine and autocrine effects on the heart through its vasoconstrictor, inotropic, chronotropic, and growth factor actions (1, 19, 25, 31). The pressor effects of Ang II are acute, readily reversible, and nonspecific for sepsis. Ang II effects on cell signaling are more long lasting and specifically may modify responses to LPS. Ang II inhibits LPS-induced iNOS in astroglial cells (8) and renal tubular cells (36). The effects of Ang II on LPS-induced iNOS activity and contractile function in adult cardiac myocytes are unknown.

We hypothesized that Ang II modulates the cardiac response to LPS. To study the direct effects of clinically relevant levels of LPS (ng/ml), adult cardiac myocytes were isolated with depyrenated digestive enzymes to minimize induction of LPS tolerance during cell isolation (23). We report that Ang II and LPS have synergistic, dose-dependent effects on endogenous NO-cGMP production that impairs excitation-contraction coupling in cardiac myocytes. The interaction between Ang II and LPS may exacerbate myocardial depression during sepsis.

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136 NaCl, 5.4 KCl, 1 MgCl2, 0.33 NaH2PO4, 10 glucose, and 5 HEPES, pH 7.4 at 37°C. The heart was perfused for an additional 20–30 min with 50 µM Ca2+. Tyrode solution containing depyrogenated (see Enzyme depyrogenation) collagenase B (lot no. 14325222, Boehringer Mannheim, Indianapolis, IN) and protease (lot no. 84H10613, Sigma Chemical, St. Louis, MO) in a 2:1 ratio. The left ventricular free wall was removed and the myocytes dispersed by gentle mechanical dissection. The cell suspension was passed through a 250-µm nylon filter to remove undigested myocytes and connective and fatty tissues. The myocytes were rinsed with serial washes, gradually increasing the Ca2+ concentration to 2 mM. Cardiac myocytes were stored at 22°C in MEM (GIBCO, Grand Island, NY) supplemented with 3% autologous serum taken from the same rabbit. Serum was added to supply LPS binding protein and soluble CD14 receptors. These enhance the response to LPS, particularly in cells that lack the membrane form of CD14. The cardiac myocytes in this preparation lack evidence of CD14 (39). In preliminary studies, serum enhanced LPS effects on cardiac myocytes through a CD14-mediated mechanism (22).

Enzyme depyrogenation. The original collagenase and protease enzymes used for cell isolation contained 100–300 ng/ml LPS. To minimize exposure of the heart to LPS contaminants which induce LPS tolerance, the enzymes were depyrogenated by a series of Triton X-114 and polymyxin B washes (23). The enzymes were dissolved in nominally Ca2+-free Tyrode solution supplemented with Triton X-114 (1:100 volume) and stirred on ice for 2 h. The solution was warmed to 37°C to separate the Triton X-114 layer and centrifuged to recover the supernatant. Polymyxin B was added to the supernatant (1:100 volume), stirred at 22°C for 2 h, warmed to 37°C, and centrifuged to recover the supernatant. The solution was passed through a column of pyrogen-free SM-4 biobeads (Bio-Rad Laboratories, Richmond, CA) to remove residual detergent. LPS levels in the depyrogenated enzymes were 0.3–0.7 ng/ml, which minimizes the development of LPS tolerance during cell isolation (23).

Measurements of cell contraction. The cells were plated on 2-mL superfusion chambers (Biopetchs, Butler, PA) and continuously perfused with 2 mM Ca2+-free Tyrode solution at 22°C with flow rate of 1 mL/min using a syringe pump (Harvard Apparatus, South Natick, MA). Myocytes were stimulated at 0.5 Hz with platinum electrodes connected to a stimulator (S44, Grass Instruments, Quincy, MA). The cells were visualized by an inverted microscope (Nikon Diaphot, Tokyo, Japan). Cell images, recorded by a solid-state camera (GP-CD60, Panasonic, Secaucus, NJ) attached to the microscope, were processed by a video-edge detection system (Crescent Electronics, Sandy, UT) to measure cell length. Data were sampled at 120 Hz with on-line analog-to-digital conversion using a 486 computer with a DI-220 Codas Data Acquisition System and WINDAQ software (Dataq Instruments, Akron, OH). Five consecutive steady-state beats were averaged to measure myocyte length at rest (Lmax), minimum cell length (Lmin), percent cell shortening [100 × (Lmax – Lmin)/Lmax], peak rate of cell shortening (–dL/dt), and peak rate of cell lengthening (+dL/dt).

Measurements of Ca2+ fluorescence by indo 1. The myocytes were loaded with 6 µM indo 1-AM (Molecular Probes, Eugene, OR) solubilized in DMSO containing Pluronic F-127 (Molecular Probes) at 22°C. After 15 min, the cells were rinsed to remove extracellular dye. The myocytes were stimulated to contract at 0.5 Hz while superfused with 2 mM Ca2+-free Tyrode solution.

Indo 1 fluorescence was measured with a PTI Alphalascan system (Photon Technology International, South Brunswick, NJ), as previously described (39). Indo 1 fluorescence was excited at 360 nm and detected at 405 and 485 nm by photomultiplier tubes. The emission field was restricted to a single myocyte with an adjustable window. Background fluorescence from a similar-sized field was subtracted from myocyte signals before calculating fluorescence ratios (405/485).

The system was modified to simultaneously measure cell lengths with Ca2+ fluorescence. A 650-nm light-emitting diode light was used to transilluminate the cells. The cell image under this red light was recorded with the solid-state camera and processed with the video-edge detection system to measure cell length. Ca2+-free fluorescence and cell lengths were sampled at 120 Hz with on-line analog-to-digital conversion using a 486 computer with a PTI Alphalascan system (OSCAR) and FeliX software (Photon Technology International). Five consecutive steady-state beats were averaged to measure Lmax, Lmin, percent cell shortening, and peak ±dL/dt. From the Ca2+ fluorescence data, we measured the diastolic fluorescence ratio (Rd), peak systolic fluorescence ratio (Rs), amplitude of the transient (Amp = Rs – Rd), time to 90% decay of the transient (T90), and the integral of the transient above the diastolic level (lR).

Intracellular Ca2+ calibration. Immediately after recording fluorescence intensities at 405 and 485 nm, cells were superfused with the same buffer supplemented with 2.3-butanedione monoxide (40 mM, Sigma Chemical) and the nonfluorescent Ca2+ ionophore BrA-23137 (10 µM, Molecular Probes) to measure the maximum value of the fluorescence ratio (Rmax). The cells were then superfused with nominally Ca2+-free buffer with 10 mM EGTA to measure the minimum value of the fluorescence ratio (Rmin). The concentration of intracellular free Ca2+ ([Ca2+]i) was estimated by the equation of Grynkiewicz et al. (12) as follows: [Ca2+]i = Kd × β × (R – Rmin)/(Rmax – R), where Kd is the dissociation constant for indo 1 (taken to be 250 nM), β is the ratio of free to bound indo 1 fluorescence at 485 nm, and R is the ratio of two fluorescence intensities measured at 405 and 485 nm. There was no difference in Rmin or Rmax values between control and LPS-treated myocytes.

To assess the degree of compartmentation, indo 1-loaded myocytes were superfused with nominally Ca2+-free Tyrode solution containing digitonin (50 µM, Sigma Chemical). The fluorescence intensities at both wavelengths declined to zero dye (background) levels within 3 min, with a similar time course in control and LPS-treated cardiac myocytes. These results suggest that the majority of the intracellular indo 1 fluorescent dye was within the cytosol and that LPS did not alter the degree of dye compartmentation. The calculated resting and peak [Ca2+]i values were 285 ± 22 and 647 ± 31 (SE) nM, respectively (n = 6 myocytes), comparable to our prior study (39).

cGMP measurements. Cardiac cGMP levels were measured in myocytes after 1- or 6-h incubation. For the last 20 min of the incubation period, IBMX (1 mM, Sigma Chemical), a phosphodiesterase inhibitor, was added to the cell culture to inhibit cGMP breakdown. The media were removed, and the cells were lysed with ice-cold 65% ethanol. The supernatants were recovered after centrifugation and dried in a SpeedVac System (Savant Instruments, Farmingdale, NY). The cGMP content of cell extracts was determined by enzyme immunoassay after acetylation using the Biotrak system (Amersham Life Science, Arlington Heights, IL). The cGMP content was normalized to milligrams of protein, which was determined by a dye-binding assay (Pierce Chemical, Rockford, IL) with BSA used as a standard.
ANG II EXACERBATES LPS-INDUCED CARDIAC DEPRESSION

Study protocols. The interaction between ANG II and LPS was evaluated by incubating cardiac myocytes with or without 10 ng/ml LPS (Escherichia coli 055: LPS no. B5 lot no. 2039F, List Biological Laboratories, Cambell, CA), with or without 100 nM ANG II (Sigma Chemical) at 22°C. Measurements were obtained within the first hour and 6 h after incubation. Cell contractions were measured 10–15 min after perfusing myocytes with 2 mM Ca²⁺ Tyrode solution (without ANG II). This minimized the acute positive inotropic effects of ANG II, which are largely abolished within 5 min after ANG II washout (15).

The dose response of ANG II effects was evaluated by coincubating cardiac myocytes with 10 ng/ml LPS and ANG II in concentrations ranging from 10⁻¹² to 10⁻⁶ M. Cell contractions were measured after 6-h incubation. The relationship between percent cell shortening and ANG II dose was fit to a sigmoidal curve using a software program (Prism, GraphPad Software, San Diego, CA).

The specificity of ANG II receptor subtypes was assessed by coincubating cardiac myocytes with 10 ng/ml LPS and 100 nM ANG II, with or without 1 µM PD-123319 (provided by Parke-Davis, Ann Arbor, MI), an ANG II type 2 receptor (AT₂) antagonist. Cell contractions were measured after 6-h incubation. In a separate protocol, cGMP, a second messenger of NO, was measured in cardiac myocytes incubated with or without 10 ng/ml LPS, with or without 100 nM ANG II. cGMP was measured after 1- and 6-h incubations.

The role of NO pathways in mediating ANG II effects was evaluated by incubating cardiac myocytes with 10 ng/ml LPS and 100 nM ANG II, with or without 1 mM NOG-monomethyl-L-arginine (L-NAME; Calbiochem-Novabiochem, San Diego, CA), a NO synthase inhibitor. Cell contractions were measured after 6-h incubation. In a separate protocol, cGMP, a second messenger of NO, was measured in cardiac myocytes incubated with or without 10 ng/ml LPS, with or without 100 nM ANG II. cGMP was measured after 1- and 6-h incubations.

The specificity of ANG II receptor subtypes was assessed by coincubating cardiac myocytes with 10 ng/ml LPS and 100 nM ANG II, with or without 1 µM DuP-753 (provided by Du Pont-Merck Pharmaceutical, Wilmington, M1), an ANG II type 1 receptor (AT₁) antagonist. Cell contractions were measured after 6-h incubation. In another protocol, myocytes were incubated for 6 h with 10 ng/ml LPS and 100 nM ANG II, with or without 1 µM PD-123319 (provided by Parke-Davis, Ann Arbor, MI), an ANG II type 2 receptor (AT₂) antagonist.

To evaluate ANG II and LPS effects on excitation-contraction coupling, cardiac myocytes were loaded with indo 1-AM to simultaneously measure Ca²⁺ transients and cell length 6 h after incubation with or without 10 ng/ml LPS and with or without 100 nM ANG II.

Colorimetric MTS assay. To assess mitochondrial activity in cardiac myocytes, a tetrazolium assay was performed by using CellTiter 96 AQueous (Promega, Madison, WI) composed of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) and phenazine methosulfate (PMS). MTS is a tetrazolium salt and aqueous-soluble analog of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (10, 20). The conversion of tetrazolium into formazan by enzymes in the electron transport chain is an indicator of mitochondrial activity. Cardiac myocytes were incubated for 6 h, rinsed, and diluted with culture media for a final concentration of 1,250 cells/ml. The MTS/PMS solution (20:1 volume) of 20 µl was introduced to each well of a 96-well microplate, containing 100 µl of cell suspension. The plates were incubated at 37°C for 4 h, placed on a plate shaker for 30 s (VorTemp 56, National Labnet, Woodbridge, NJ), then read on a plate reader (Vmax, Molecular Devices, Menlo Park, CA) using SOFTmax software (Molecular Devices). Optical density was measured at 492 nm with a reference wavelength of 690 nm.

Statistics. Comparisons among the groups were carried out by ANOVA. When a significant difference among groups was found, multiple pairwise t comparisons between individual groups were performed using the Student-Newman-Keuls method. In all cases, differences were considered significant at P < 0.05. Data are presented as means ± SE.

RESULTS

ANG II effects on the contractile response to LPS in cardiac myocytes. Figure 1 shows percent cell shortening in cardiac myocytes incubated with or without 10 ng/ml LPS and with or without 100 nM ANG II. Neither LPS nor ANG II affected cell shortening within the first hour of incubation. However, after 6 h, LPS depressed cell shortening compared with control myocytes. ANG II alone did not affect cell shortening. ANG II coincubation with LPS exacerbated LPS-induced contractile

Table 1. Peak rates of cell shortening and lengthening after LPS (10 ng/ml) and/or ANG II (100 nM)

<table>
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<tr>
<th></th>
<th>Control</th>
<th>LPS</th>
<th>ANG II</th>
<th>LPS + ANG II</th>
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<tr>
<td>&lt;1 h</td>
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<tr>
<td>- dL/dt, µm/s</td>
<td>-154.6 ± 5.9 (15)</td>
<td>-161.5 ± 5.1 (15)</td>
<td>-158.1 ± 5.7 (15)</td>
<td>-158.5 ± 5.5 (15)</td>
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<tr>
<td>+ dL/dt, µm/s</td>
<td>183.6 ± 6.8 (15)</td>
<td>177.1 ± 5.3 (15)</td>
<td>178.6 ± 5.2 (15)</td>
<td>171.4 ± 6.9 (15)</td>
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<td>6 h</td>
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<tr>
<td>- dL/dt, µm/s</td>
<td>-135.3 ± 5.5 (20)</td>
<td>-105.9 ± 5.1* (20)</td>
<td>-149.3 ± 7.3 (20)</td>
<td>-82.6 ± 4.5†‡ (21)</td>
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<tr>
<td>+ dL/dt, µm/s</td>
<td>169.8 ± 5.5 (20)</td>
<td>142.3 ± 5.4* (20)</td>
<td>177.6 ± 6.8 (20)</td>
<td>113.2 ± 4.5†‡ (21)</td>
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Values are means ± SE of number of myocytes given in parentheses. Lipopolysaccharide (LPS) was used at 10 ng/ml, and ANG II was used at 100 nM. ± dL/dt, rate of cell lengthening; –dL/dt, rate of cell shortening. *P < 0.05 vs. control. †P < 0.05 vs. LPS alone. ‡P < 0.05 vs. ANG II alone. Statistical significance was determined by ANOVA.
depression. Table 1 shows similar results with peak rates of cell shortening (−dL/dt) and cell lengthening (+dL/dt).

Figure 2 shows the ANG II dose-response relationship in cardiac myocytes exposed for 6 h to 10 ng/ml LPS with ANG II doses ranging from 1 pM (10^{-12} M) to 1 µM (10^{-6} M). As a reference response, cell shortening was 18.0 ± 0.4% in control myocytes. Cell shortening decreased to 15.8 ± 0.4% with 10 ng/ml LPS (without ANG II). ANG II caused dose-dependent exacerbation of LPS-induced contractile depression. The ANG II dose-response data fit a sigmoidal curve with r^2 = 0.998. Coincubation of myocytes with LPS and the highest dose of ANG II tested (1 µM) reduced cell shortening to 10.3 ± 0.4%, nearly a fourfold increase in severity of contractile depression compared with LPS alone. The EC_{50} value for this relationship was 5.3 nM, which is comparable to the K_d value (4.5 nM) for rabbit left ventricular myocardial ANG II receptors (2).

Role of NO-cGMP pathways in ANG II effects. To evaluate the role of NO in mediating ANG II effects, cardiac myocytes were incubated with or without 10 ng/ml LPS, 100 nM ANG II, and 1 mM L-NMMA, a NO synthase inhibitor. Figure 3 shows cell shortening after 6 h. As in the preceding protocols, LPS depressed cell function, which was exacerbated by coincubation with ANG II. L-NMMA alone had no effect on cell shortening. However, L-NMMA completely blocked ANG II exacerbation of LPS effects, suggesting a NO-mediated mechanism.

cGMP, a second messenger of NO, was measured in cardiac myocytes incubated with or without 10 ng/ml LPS and with or without 100 nM ANG II. After 1 h, cardiac cGMP in control myocytes (298.8 ± 37.2 fmol/mg protein) was unaffected by LPS (315.0 ± 43.2 fmol/mg protein), ANG II (279.6 ± 42.6 fmol/mg protein), or LPS combined with ANG II (304.8 ± 43.8 fmol/mg protein) (n = 4 in each group, P = NS). Figure 4 shows cardiac cGMP levels after 6 h of incubation. Cardiac cGMP was higher in LPS-exposed than in control myocytes. ANG II alone had no effect on cGMP. However, ANG II combined with LPS markedly increased cGMP levels in cardiac myocytes. These findings suggest that ANG II enhanced LPS-induced iNOS activity in cardiac myocytes.

Role of ANG II receptor subtypes. To evaluate the type of angiotensin receptors involved in this response, cardiac myocytes were incubated with or without 10 ng/ml LPS, 100 nM ANG II, and 1 µM DuP-753 (an AT_1 antagonist). Figure 5 shows cell shortening after 6 h. LPS induced cell depression, which was exacerbated by coincubation with ANG II. Coincubation with DuP-753 completely abolished ANG II exacerbation of LPS effects, restoring cell shortening to a level comparable to LPS alone. DuP-753 alone had no effect. In a similar protocol, we evaluated the effects of PD-123319, an AT_2 antagonist. In contrast to DuP-753, PD-123319...
provided no protective effects against ANG II exacerbation of LPS effects (data not shown). Blockade of ANG II exacerbation of LPS effects by DuP-753, but not by PD-123319, indicates that ANG II effects were mediated through AT1 and not by AT2 receptors.

ANG II and LPS effects on Ca$^{2+}$ transients in cardiac myocytes. The effects of LPS and ANG II on excitation-contraction coupling were evaluated with simultaneous cell shortening and Ca$^{2+}$ transients measurements in cardiac myocytes after the 6-h incubation. Figure 6 shows representative tracings in indo 1-AM-loaded cardiac myocytes, and Table 2 presents the group data. LPS depressed cell shortening, peak $-\Delta L/dt$, and $+\Delta L/dt$ without any significant alteration in Ca$^{2+}$ transients. ANG II did not affect contractile function or Ca$^{2+}$ transients. ANG II exacerbated LPS-induced contractile depression, with a more severe depression in cell shortening, peak $-\Delta L/dt$, and $+\Delta L/dt$. This was accompanied by significant changes in Ca$^{2+}$ transients with an elevated $R_d$, decreased Amp, prolongation of $T_{90}$, and decrease in the InR. Changes in Ca$^{2+}$ transients were restored by 1 mM L-NMMA (data not shown).

ANG II and LPS effects on mitochondrial function in cardiac myocytes. The effects of LPS and ANG II on mitochondrial activity were assessed with a MTS assay in cardiac myocytes incubated with or without 10 ng/ml LPS, 100 nM ANG II, and 1 mM L-NMMA. Figure 7 shows that after 6 h of incubation, there was no change in mitochondrial activity with LPS alone but a significant decrease in myocytes treated with LPS and ANG II. This was completely restored by 1 mM L-NMMA, suggesting that the reduction of mitochondrial activity is caused by activation of NO synthase. This was not related to differences in cell viability, since the percentage of rod-shaped cells was similar ($\pm 61 \pm 2\%$) in each of the five groups.

DISCUSSION

This study demonstrated a direct interaction between ANG II and LPS in inducing contractile depression in cardiac myocytes through NO-cGMP-dependent...
pathways. LPS (10 ng/ml) alone increased cardiac cGMP production after 6 h (but not 1 h), which depressed cell shortening without altering Ca$^{2+}$ transients. ANG II (100 nM) alone had no effect. However, coincubation of ANG II with LPS doubled the increase in cardiac cGMP and exacerbated contractile depression three- to fourfold, compared with LPS alone. This was associated with elevated resting Ca$^{2+}$ levels, depressed amplitude of Ca$^{2+}$ transients, slower decline of Ca$^{2+}$ transients, and impaired mitochondrial function, none of which occurred with ANG II or LPS alone. Thus ANG II and LPS have synergistic effects that impair excitation-contraction coupling at multiple sites. ANG II exacerbation of LPS effects was ANG II dose dependent, completely blocked by L-NMMA (a NO synthase inhibitor), and mediated through AT1 receptors (blocked by the AT1 receptor antagonist DuP-753).

LPS has pleiotropic effects on a variety of cells that are important at the low nanogram per milliliter LPS levels found in clinical sepsis (6). In cardiac myocytes, low levels of LPS induce iNOS (21), depress contractility (34), and depress myofilament responsiveness to Ca$^{2+}$ (39). Although low levels of LPS depress baseline contractility modestly, there is a marked impairment in contractile reserve that severely attenuates the contractile response to β-adrenergic stimulation (39).

The current study focused on interactions between ANG II and LPS effects. LPS activates the renin-angiotensin system to increase circulating angiotensinogen (protein precursor of ANG I), renin, and ANG II levels (4, 9, 32). LPS also activates the local renin-angiotensin system in several tissues, including the heart (16, 19, 30). Activation of the cardiac renin-angiotensin system may affect cardiac myocytes in a paracrine or autocrine manner, independently of circulating ANG II (1, 25, 40).

ANG II affects cell signaling events and protein synthesis (1, 11, 25, 31), which may modulate the cardiac response to LPS. LPS depresses contractility by inducing iNOS in cardiac myocytes to increase NO and cGMP after 6 h (but not 1 h) and blockade of ANG II effects with L-NMMA. ANG II exacerbated LPS-induced contractile depression over a range of ANG II doses from 1 nM to 1 µM, with an EC$_{50}$ of 5.3 nM (Fig. 2). These levels are relevant, since ANG II concentrations are ~0.1 nM in rabbits (24), and LPS increases by severalfold both circulating and tissue levels of angiotensinogen and ANG II (4, 9, 16, 19, 30). ANG II exacerbated LPS effects through AT1 receptors that
were blocked by DuP-753, whereas PD-123319, a selective AT2 receptor antagonist, had no effect. This is similar to most cardiac effects of ANG II, which are mediated through AT1 receptors (11).

Endogenously generated NO and cGMP induced dose-dependent impairments in excitation-contraction coupling. A modest increase in cGMP (with LPS alone) depressed contractile function without altering Ca2+ transients, indicating decreased myofilament responsiveness to Ca2+ (39). Higher levels of cGMP induced by ANG II with LPS depressed contractile function more severely in association with impaired Ca2+ handling (Table 2). We did not observe an increase in contractility, which some have reported with low levels of exogenous NO donors. This may reflect differences in kinetics and/or compartmentalization effects with endogenously generated NO, compared with exogenous NO donors.

NO can inhibit mitochondrial respiration, affecting cardiac contractility (37). We evaluated mitochondrial function in this model by using a tetrazolium assay sensitive to several steps along the electron transport chain (10, 20). As shown in Fig. 7, mitochondrial function was not altered by LPS alone but was depressed in myocytes coinubated with ANG II and LPS through a NO-mediated mechanism (blocked by L-NMMA). ANG II and LPS may generate higher endogenous NO levels than with LPS alone to inhibit mitochondrial function consistent with prior studies (37). Several mitochondrial enzymes contain non-heme iron sulfur clusters (mitochondrial aconitase, complex I and complex II of the mitochondrial electron transport chain) or heme groups (cytochrome-c oxidase or complex IV) that are potential target sites for the actions of NO. Moreover, NO induces free radicals such as peroxynitrite (ONOO−), which inhibit complex I and complex II in cardiac mitochondria (7, 29). NO-induced suppression of mitochondrial energy metabolism may contribute to alterations in Ca2+ handling and contractile function. There is a complex interdependence between these factors, which cannot be evaluated by this study design. Thus it is difficult to determine the extent to which mitochondrial inhibition contributes to myocyte contractile dysfunction from the current data. Further studies are needed to address this important issue.

In conclusion, ANG II enhanced LPS-induced production of endogenous NO and cGMP, which impaired cardiac excitation-contraction coupling in a dose-dependent manner. The direct interaction between ANG II and NO pathways may have detrimental, long-lasting effects on cardiac myocytes that contribute to sustained myocardial depression in sepsis.

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