Role of cyclooxygenase in ventricular effects of adrenomedullin: is adrenomedullin a double-edged sword in sepsis?

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Mittra, Shivani, Jean-Marc Hyvelin, Qixian Shan, Fai Tang, and Jean-Pierre Bourreau. Role of cyclooxygenase in ventricular effects of adrenomedullin: is adrenomedullin a double-edged sword in sepsis? Am J Physiol Heart Circ Physiol 286: H1034–H1042, 2004; 10.1152/ajpheart.00337.2003.—Adrenomedullin (ADM) is upregulated in cardiac tissue under various pathophysiological conditions. However, the direct inotropic effect of ADM on normal and compromised cardiomyocytes is not clear. In rat ventricular myocytes, ADM produced an initial (<30 min) increase in cell shortening and Ca\(^{2+}\) transient and, on prolonged incubation (>1 h), a marked decrease in cell shortening and Ca\(^{2+}\) transient. Both effects were sensitive to inhibition by the ADM antagonist ADM-(22–52). The increase and decrease in cell shortening and Ca\(^{2+}\) transient were attenuated by pretreatment with indomethacin [a nonspecific cyclooxygenase (COX) inhibitor], nimesulide and SC-236 (specific COX-2 inhibitors), and tranylcypromine (a prostacyclin synthase inhibitor); SQ-29548 (a thromboxane receptor antagonist) was without effect. Cells isolated from LPS-treated rats that were in the late, hypodynamic phase of septic shock also showed a marked decrease in cell shortening and Ca\(^{2+}\) transient. Because ADM is overexpressed in sepsis, we repeated the above protocol in cells isolated from LPS-treated rats. At 4 h after LPS injection, ADM levels markedly increased in plasma, ventricles, and freshly isolated ventricular myocytes. Decreases in cell shortening and Ca\(^{2+}\) transient in LPS-treated cells were reversed by pretreatment with ADM-(22–52). Anti-ADM (rat) IgG also reversed the decrease in cell shortening and other parameters of cell kinetics. Indomethacin, SC-236, and tranylcypromine restored cell contractility and the decrease in Ca\(^{2+}\) transient, whereas SQ-29548 had no effect, implying that prostacyclin played a role in both effects. However, with regard to cell-shortening kinetics, indomethacin and SQ-29548 decreased the amount of time taken by the cells to return to baseline, whereas SC-236 and tranylcypromine did not, implying that not only prostacyclin, but also thromboxane, is involved. The results indicate that ADM interacts with COX to yield prostanoids, which mediate its negative inotropic effect in LPS-treated rat ventricular myocytes.

ADRENOMEDULLIN (ADM)-(1–52), a 52-amino-acid-residue peptide, was first isolated from human pheochromocytoma (19). The distribution of ADM transcripts is largely restricted to heart, lungs, adrenal glands, and vascular endothelial cells (38). Recent studies have shown that ADM is upregulated under various pathophysiological conditions, such as myocardial ischemia (33), systemic inflammatory response syndrome (44), hemorrhagic and endotoxic shock (7, 34), chronic hypotension in hemodialysis (4), cirrhosis (21), and pulmonary hypertension (18). Among the noninvasive variables, plasma ADM has been proposed to be an independent predictor of mortality after acute myocardial infarction, inasmuch as plasma ADM levels, which increased significantly 2 days after the attack, were strongly associated with long-term mortality (30).

ADM may be acting as an autocrine or a paracrine factor in the regulation of cardiac function, inasmuch as ADM immunoreactivity, expression of mRNA for ADM, and abundant binding sites for ADM have been detected in the heart (16, 17). ADM is normally classified as a hypotensive peptide and a compensatory factor involved in some of the above-mentioned disorders. Generally, ADM is looked on and reported as a positive inotropic peptide (13, 37). However, the direct inotropic effect of ADM at the cardiac level is not clear. In some reports, it does not appear to have any inotropic action (32, 43). In other reports, it has been observed to produce a negative inotropic effect, namely, in isolated human ventricular myocytes (29), rabbit ventricular myocytes (15), and isolated rat heart (35). In addition to the negative inotropic effect in rabbit ventricular myocytes, ADM has been simultaneously observed to decrease intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) and L-type Ca\(^{2+}\) current (\(I_{\text{Ca,L}}\)) (15). Recently, inhibitory effects of ADM have been shown on \(I_{\text{Ca,L}}\) in guinea pig ventricular myocytes, the inhibition being mediated by a specific ADM receptor (5).

In our laboratory, a dual inotropic effect in response to ADM, specific to inhibition by its antagonist ADM-(22–52), has been observed in adult rat ventricular myocytes (12). cAMP-dependent positive inotropic and nitric oxide (NO)-cGMP-independent negative inotropic effects in response to ADM have been observed (12).

Because ADM is excessively produced in septic shock, we simulated the overproduction of ADM by incubating normal ventricular myocytes with a high dose of ADM for a long time. We compared the negative inotropic effect produced by long-term exposure of ventricular myocytes to ADM with that produced in myocytes isolated from LPS-treated rats (12). Results from cardiomyocytes exposed to ADM and those isolated from LPS-treated rats suggest that ADM may actually be acting as a pathological contributor to ventricular failure in the late hypodynamic phase of septic shock (12).

On having ascertained that NO does not play a role in the inotropic effects of ADM, inasmuch as these effects were insensitive to inhibition by \(N\text{-nitro-L-arginine methyl ester (L-NAME)}\) (12), we have studied whether cyclooxygenase (COX) plays a part in mediating the inotropic actions and changes in [Ca\(^{2+}\)]\(_i\), caused by ADM in adult rat ventricular...
myocytes. Inasmuch as ADM is excessively produced in septic shock (10, 42), we studied whether ADM inhibition could ameliorate the cardiodepression in myocytes isolated from an experimental model of septic shock. Recently, COX-2 was reported to be overexpressed in human septic heart and endotoxic rat heart (8, 28), so we also studied the role of COX in cell shortening and [Ca\textsuperscript{2+}]\textsubscript{i} changes in myocytes isolated from LPS-treated rats.

**MATERIAL AND METHODS**

All animal experiments were conducted in accordance with the Committee on the Use of Live Animals in Teaching and Research of the University of Hong Kong. Male Sprague-Dawley rats aged 10–15 wk and weighing 250–300 g were used in this study.

**Animal models of septic shock.** Endotoxemia was induced by an injection of LPS (10 mg/kg ip), as previously described (40). Observations of conscious rats after LPS treatment were characteristic of endotoxemia, i.e., piloerection, apathy, and diarrhea. The rats (10%) that did not exhibit these characteristics were considered LPS-resistant rats and, consequently, were not used for this study. The sham control rats were injected with saline (1 ml/kg ip).

**Cell isolation procedure.** \(\text{Ca}^{2+}\) concentration and cell shortening were measured on freshly isolated ventricular myocytes. Enzymatically isolated ventricular myocytes were obtained using the procedure previously described (40). After \(\text{Ca}^{2+}\) reconstitution, the yield of viable myocytes was 45–50%. The final cell suspension contained a negligible number of nonmyocytes. Cells were maintained in a \(\text{Ca}^{2+}\)-containing HEPES-buffered solution (HBS) consisting of (in mM) 130 NaCl, 5.6 KCl, 1.25 CaCl\textsubscript{2}, 1 MgCl\textsubscript{2}, 10 glucose, 4 NaHCO\textsubscript{3}, and 10 HEPES, pH 7.4. The cells were used within 6 h of incubation. The average resting cell length was 116.54 ± 3.29 \(\mu\)m for normal control cells (n = 70) and 112.0 ± 1.7 \(\mu\)m for LPS-treated cells (n = 83), and the difference was not significant. In all experiments, a cell length of >100 \(\mu\)m was chosen as a prerequisite for cell selection. \([\text{Ca}^{2+}]_i\), was measured at 24–26°C, and the myocyte contraction was observed at 32°C.

**[Ca\textsuperscript{2+}]\textsubscript{i} measurements.** \([\text{Ca}^{2+}]_i\), changes in paced ventricular myocytes were monitored fluorometrically using the \(\text{Ca}^{2+}\)-sensitive probe fura 2 (1 \(\mu\)M). The recording system included an inverted microscope (Axiolabor S170, Zeiss) fitted with epifluorescence (monochromator TILL Polychrome II). The cells were alternately illuminated at 340 and 380 nm at a frequency of 140 Hz. Emission for each excitation wavelength was filtered at 510 nm and digitized using interface (EPC9, HEKA). The ratio of fluorescence at 340 nm to fluorescence at 380 nm \((F_{340/380})\) was displayed online using Xchart software.

**Measurement of myocyte contraction.** Contractile parameters of ventricular myocytes were assessed by a video-based edge-detection system (IonOptix, Milton, MA) that sampled cell length at 60 Hz. Briefly, the cells were placed in a chamber mounted on the stage of an inverted microscope (Nikon, Tokyo, Japan). The cells were field stimulated (model S88, Grass) with 20% suprathreshold voltage at a frequency of 0.2 Hz (5-ms duration) with a platinum electrode. The myocyte being studied was displayed on the computer monitor with the help of an IonOptix MyoCam charge coupled device camera, which was attached to the sidearm of the microscope. SoftEdge acquisition software (IonOptix) captured and converted the changes in cell length to digital signals. The signals were analyzed by IonWizard analysis software (IonOptix) to obtain the contractile parameters, including the cell kinetics. Cell shortening was expressed as a percentage of resting cell length. The cells were incubated in different media (control and in the presence of drugs) for 30 min before stimulation. Data were recorded in steady-state conditions in each experimental medium. All the inhibitors tested had no effect per se (data not shown).

**Cell shortening kinetics.** Mechanical properties of the contracting cells were analyzed using IonWizard analysis software. The following parameters were analyzed: maximal velocity of shortening \((\pm \text{d}L/\text{dt})\) in \(\mu\text{m/s}\), peak height (peak baseline height, in \(\mu\)m), time to peak (i.e., the time peak occurred relative to the transient time measured in s), maximal velocity of relengthing \((-\text{d}L/\text{dt})\) in \(\mu\text{m/s}\), and time taken to return to base length (i.e., time required for the cell shortening transient to return to 90.0% of peak during the recovery phase of the transient, in s).

**Hemodynamic measurements.** Rats were anesthetized with an injection of urethane (1.2 g/kg ip), a rectal probe was inserted, and body temperature was maintained at 37°C. The trachea was cannulated to facilitate respiration. The right femoral artery was cannulated and connected to a blood pressure transducer (model MLT1050, ADInstruments). The signal for systemic arterial blood pressure was recorded in a personal computer via an analog-to-digital converter (PowerLab/410, ADInstruments). After a period of stabilization (~30 min), LPS (10 mg/kg) or PBS (1 ml/kg) was injected intraperitoneally. Blood pressure was monitored for 6 h. The animals were under the effect of urethane anesthesia throughout the experiment.

**ADM assay.** RIA of ADM for the plasma samples was performed using the method described by Hwang and Tang (11). Briefly, blood samples from sham control and LPS (10 mg/kg)-pretreated rats were collected in chilled tubes containing EDTA and immediately centrifuged at 4,000 rpm at 4°C for 20 min. The plasma was decanted and kept at −70°C until analysis. Plasma was extracted on a C\textsubscript{18} Sephadex C. Then 100 \(\mu\)l of normal rabbit serum, and 100 \(\mu\)l of polyethylene glycol were added, and the samples were incubated for 3 h at 4°C. The samples were then centrifuged at 4,000 rpm at 4°C for 1 h. The supernatant was aspirated off, and radioactivity of the pellet was measured on a gamma counter. The assay was performed in duplicate. The rat ADM assay has no cross-reactivity with human ADM, amylin, or endothelin peptides. For determination of ADM levels in rat ventricles, the myocytes were isolated and the samples from sham control and LPS (10 mg/kg)-pretreated rats were collected in chilled tubes containing EDTA and immediately centrifuged at 4,000 rpm at 4°C for 20 min; 100 \(\mu\)l of sample were incubated with 100 \(\mu\)l of ADM antisera and \(^{125}\text{I}\)-labeled ADM overnight at 4°C. Then 100 \(\mu\)l of secondary antibody (goat anti-rabbit IgG serum), 100 \(\mu\)l of normal rabbit serum, and 100 \(\mu\)l of polyethylene glycol were added, and the samples were incubated for 3 h at 4°C. The samples were then centrifuged at 4,000 rpm at 4°C for 1 h. The supernatant was aspirated off, and radioactivity of the pellet was measured on a gamma counter.

**Data analysis.** The response of each cell to stimulation was determined by averaging 10 successive transients in the steady-state condition. \(\text{Ca}^{2+}\) transients are expressed as percent changes from resting \(F_{340/380}\) in each cell or as \(\Delta F_{340/380}\) (percent control). Cell shortening is expressed as percent change in resting cell length. Values are means ± SE of n cells. Differences between two group means were evaluated by unpaired Student’s \(t\)-test. Comparisons between multiple groups were made by one-way analysis of variance with a post hoc Newman-Keuls \(t\)-test. \(P < 0.05\) was considered significant.

**Materials.** Fura 2-AM was purchased from Molecular Probes (Eugene, OR); human ADM–(1–52), human ADM–(22–52), and anti-
ADM (rat) IgG from Peninsula Laboratories (San Carlos, CA); SQ-29548 from Alexis; SC-236 from Calbiochem; and indomethacin, nimesulide, tranylcypromine, LPS (Escherichia coli serotype 0111: B4), modified Eagle's medium, collagenase (type I), bovine serum albumin, and all other chemicals from Sigma-Aldrich (St. Louis, MO).

RESULTS

Myocytes from control cells. In ventricular myocytes from control rats, pretreatment with 100 nM ADM-(1–52) for up to 1 h did not change significantly the resting value of F340/380 [0.62 ± 0.02 (n = 55) vs. 0.65 ± 0.02 (n = 55)]. Within 30 min, pretreatment of cells with ADM-(1–52) induced a significant increase in Ca$^{2+}$ signal induced by field stimulation (Fig. 1A). After 1 h, pretreatment with 100 nM ADM-(1–52) induced a significant decrease in Ca$^{2+}$ response (Fig. 1B). In myocytes from control rats, ADM-(1–52) produced a significant increase in cell shortening on pretreatment with 1 nM ADM within 30 min and a marked decrease in cell shortening at >1 h (Fig. 1C). The positive and negative inotropic responses resulted from activation of ADM receptors, inasmuch as 100 nM ADM-(22–52), an antagonist of ADM-(1–52), fully abolished them (Fig. 1C).

Pretreatment with 10 μM indomethacin, a nonspecific blocker of COX-1 and COX-2, abolished the positive and negative inotropic effects of ADM (Fig. 2, A and C). A similar effect was observed with 10 μM nimesulide, a specific COX-2 inhibitor (Fig. 2, A and C). Pretreatment with 100 μM tranylcypromine, a prostacyclin synthase inhibitor, also abolished the positive and negative effects of ADM, whereas 10 μM SQ-29548, a thromboxane receptor antagonist, had no effect (Fig. 2, B and D). Indomethacin (25 μM), 10 μM SC-236 (another specific COX-2 inhibitor), and 100 μM tranylcypromine abolished the increase and decrease in [Ca$^{2+}$], induced by 1 nM ADM (Fig. 3). SQ-29548 (10 μM) had no such effect on Ca$^{2+}$ transients (Fig. 3, B and D).

Effect of LPS injection on mean arterial blood pressure in anesthetized rats. Rats were randomly distributed between two groups of eight animals each. They were anesthetized, and the right femoral artery was cannulated for blood pressure measurement. The baseline values of mean arterial blood pressure before saline or LPS injection were not significantly different between the two groups of animals. Mean arterial blood pressure was significantly lower in rats injected with LPS (10 mg/kg ip) than in sham-operated animals from 3 h after LPS injection until the end of the recording, i.e., 6 h after LPS injection (Fig. 4A). Usually, LPS injection caused a transient fall in mean arterial blood pressure that returned to or above baseline within 2 h. Mean arterial blood pressure then gradually fell over the next 4 h; then the rats were killed.

Myocytes from LPS-treated rats. Resting F340/380 was significantly decreased in ventricular myocytes isolated from rats killed 4 h after LPS injection compared with normal myocytes [0.54 ± 0.01 (n = 99) vs. 0.61 ± 0.01 (n = 154), P < 0.05]. The amplitude of the Ca$^{2+}$ transient elicited in myocytes isolated from LPS-treated rats by electrical field stimulation was significantly decreased compared with transients obtained in myocytes isolated from control rats (Fig. 5A). In cardiomyocytes from LPS-treated rats, application of ADM-(1–52) had no effects on Ca$^{2+}$ transient (data not shown). In contrast, 100 nM ADM-(22–52), an ADM receptor antagonist, induced a significant increase in Ca$^{2+}$ transient (Fig. 5A).

![Fig. 1. Effect of adrenomedullin (ADM)-(1–52) on Ca$^{2+}$ transient and cell shortening in myocytes isolated from control rats. Positive (A) and negative (B) effects were induced by ADM-(1–52) on Ca$^{2+}$ transients recorded after cells were incubated for 30 min (A) or >1 h (B) in physiological salt solution with or without 100 nM ADM-(1–52). Values (means ± SE of 15–55 cells from 4–6 rats) are expressed as percent increase in the ratio of fluorescence at 340 nm to fluorescence at 380 nm (340/380). C: positive and negative effects of 1 nM ADM-(1–52) on cell shortening in response to electrical field stimulation in normal myocytes at 30 min and >1 h. Pretreatment with 100 nM ADM-(22–52) abolished both effects. Values are means ± SE of 21–30 cells from 4–6 rats. *P < 0.05 vs. control. **P < 0.05 vs. ADM-(1–52).]
In cells treated with LPS, cell shortening was markedly decreased (Fig. 5B), and incubation with 100 nM ADM-(22–52) or 300 nM anti-ADM (rat) IgG restored the cell contractility (Fig. 5B). Pretreatment with 25 μM indomethacin and 10 μM SC-236 significantly enhanced myocyte contractility closer to the control values (Fig. 6B). Tranycypromine (100 μM) also markedly increased cell shortening, but 10 μM SQ-29548 did not (Fig. 6D). Pretreatment with 25 μM indomethacin and 10 μM SC-236 significantly enhanced myocyte contractility closer to the control values (Fig. 6B). Tranycypromine (100 μM) also markedly increased cell shortening, but 10 μM SQ-29548 did not. *P < 0.05 vs. control. ΔP < 0.05 vs. ADM-(1–52). NS, not significant.
methacin, 10 μM SC-236 and 100 μM tranylcypromine produced a significant increase in Ca$^{2+}$ transients (Fig. 6, A and C), whereas 10 μM SQ-29548 had no significant effect (Fig. 6 C).

**Cell shortening kinetics.** LPS-treated myocytes exhibited a highly depressed peak height (39.85 ± 6.35% decrease) and $+dL/dt$ (47.66 ± 6.71% decrease), a significant increase in time to peak (15.68 ± 4.05%), significantly reduced $-dL/dt$ (62.54 ± 5.98% decrease), and a prolonged time taken to return to base length (54.92 ± 9.37% increase) with respect to normal myocytes (Table 1). The ADM antagonist ADM-(22–52) and the anti-ADM (rat) IgG reversed the changes in cell kinetics of LPS-treated rats to levels comparable to normal control cells (Table 1). Indomethacin, SC-236, and tranylcypromine pretreatment significantly increased peak height (53.15 ± 12.48, 57.85 ± 10.64, and 62.55 ± 10.71%, respectively) and $-dL/dt$ (63.34 ± 13.72, 38.72 ± 11.87, and 64.37 ± 12.16%, respectively) compared with the LPS-treated myocytes, but SQ-29548 pretreatment did not (Table 1). Among the prostanoid inhibitors, only SQ-29548 caused a significant decrease in time to peak (16.55 ± 3.30%), which was comparable to that of the normal control cells. The $-dL/dt$ was significantly enhanced with all pretreatments, namely, indomethacin, SC-236, tranylcypromine, and SQ-29548 (Table 1). The time taken to return to base length was significantly reduced by indomethacin (19.74 ± 6.30%) and SQ-29548 (31.16 ± 5.80%) pretreatment; however, SC-236 and tranylcypromine had no effect (Table 1). The time taken to return to base length with SQ-29548 pretreatment was comparable to that of normal control cells (Table 1). The cell kinetics of the rest of the inhibitors with respect to the normal control cells are shown in Table 1.

**RIA for rat ADM.** A specific RIA for rat ADM was used to estimate the presence of ADM in plasma from sham control and LPS-treated rats. After LPS treatment (10 mg/kg for 4 h), the plasma concentration of immunoreactive rat ADM strongly

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**Fig. 4.** A: effect of intraperitoneal injection of LPS on mean arterial blood pressure (MAP). Shaded bar, ADM estimated in plasma (B), rat ventricles (C), and rat ventricular myocytes (D). Open and solid bars, ADM in sham control and LPS-treated rats, respectively. Values are means ± SE; n = 5. *Significantly different from sham-operated control (P < 0.05).

**Fig. 5.** A: averaged Ca$^{2+}$ transients recorded in myocytes isolated from sham control rats and rats treated with LPS and LPS + 100 nM ADM-(22–52). Values are means ± SE from 25–54 cells from 4–6 rats. B: cell shortening in electrically stimulated rat ventricular myocytes from normal control rats and rats treated with LPS, LPS + 100 nM ADM-(22–52), and LPS + 300 nM anti-ADM (rat) IgG. Values are means ± SE of 20–42 cells from 4–6 or more rats. *P < 0.05 vs. normal control. δP < 0.05 vs. LPS.
increased compared with the concentration in sham control rats (267.0 ± 6.9 fmol/ml (n = 4) vs. 22.7 ± 1.7 fmol/ml; control plasma (n = 5), P < 0.05; Fig. 4B). Similar increases in ADM levels were observed in rat ventricles (26.74 ± 4.17 and 89.99 ± 15.65 pg/mg protein in control and LPS-treated rats, respectively, n = 5) in both groups (Fig. 4C) and in rat ventricular myocytes (66.46 ± 7.9 and 106.33 ± 13.22 pg/l × 10^6 cells in control and LPS-treated rats, respectively, n = 5, Fig. 4D).

**DISCUSSION**

The results indicate that ADM-(1–52), through the ADM-(22–52)-sensitive receptor, activates a dual signaling pathway in isolated adult rat ventricular myocytes. ADM produced a marked increase in cell shortening within 30 min accompanied by an increase in Ca^{2+} transient. On prolonged exposure of >1 h, the positive inotropic effect was no longer observed. Rather, a marked negative inotropic effect was accompanied by a decrease in Ca^{2+} transient. The ADM antagonist ADM-(22–52) prevented both effects.

In our previous study (12), the ADM-induced positive effect could be suppressed by the adenylate cyclase (AC) inhibitor SQ-22536, suggesting that it resulted from activation of an AC/cAMP-dependent pathway. Surprisingly, inhibition of the positive effect unmasked a negative effect in these cells. Moreover, when ADM induced a negative effect, pretreatment with SQ-22536 led to a further decrease in Ca^{2+} transient, suggesting that ADM may be simultaneously activating two intracellular pathways, one leading to a positive inotropic effect (AC dependent) and the other to a negative inotropic effect.

**Table 1. Cell shortening kinetics of ventricular myocytes isolated from normal or LPS-treated rats**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of Cells</th>
<th>+dL/dt, μm/s</th>
<th>Peak ht, μm</th>
<th>Peak t, s</th>
<th>−dL/dt, μm/s</th>
<th>t to 90.0% BL, s</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control</td>
<td>24</td>
<td>−203.3 ± 12.9†</td>
<td>13.5 ± 0.5</td>
<td>0.13 ± 0.005</td>
<td>185.8 ± 10.0</td>
<td>0.27 ± 0.01</td>
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<tr>
<td>Septic control</td>
<td>83</td>
<td>−106.4 ± 6.3†</td>
<td>8.1 ± 0.4</td>
<td>0.16 ± 0.003†</td>
<td>69.6 ± 5.2†</td>
<td>0.40 ± 0.01†</td>
</tr>
<tr>
<td>ADM-(22–52)</td>
<td>25</td>
<td>−192.4 ± 16.0‡</td>
<td>12.7 ± 0.9‡</td>
<td>0.14 ± 0.004‡</td>
<td>151.9 ± 11.7†</td>
<td>0.28 ± 0.02‡</td>
</tr>
<tr>
<td>Anti-ADM (rat) IgG</td>
<td>32</td>
<td>−217.9 ± 20.1‡</td>
<td>13.7 ± 0.90‡</td>
<td>0.13 ± 0.005†</td>
<td>189.8 ± 28.6‡</td>
<td>0.27 ± 0.01†</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>24</td>
<td>−173.8 ± 16.2‡</td>
<td>12.4 ± 1.1‡</td>
<td>0.16 ± 0.004‡</td>
<td>130.9 ± 13.4†</td>
<td>0.32 ± 0.01†</td>
</tr>
<tr>
<td>SC-236</td>
<td>25</td>
<td>−147.6 ± 9.4‡</td>
<td>12.8 ± 0.6‡</td>
<td>0.18 ± 0.005‡</td>
<td>105.0 ± 7.3‡</td>
<td>0.40 ± 0.02†</td>
</tr>
<tr>
<td>Tranylcypromine</td>
<td>30</td>
<td>−174.9 ± 12.6‡</td>
<td>13.2 ± 0.8‡</td>
<td>0.16 ± 0.004§</td>
<td>124.7 ± 10.5‡</td>
<td>0.36 ± 0.02§</td>
</tr>
<tr>
<td>SQ 29548</td>
<td>28</td>
<td>−118.4 ± 11.8§</td>
<td>8.1 ± 0.8§</td>
<td>0.13 ± 0.005†</td>
<td>89.0 ± 10.0‡</td>
<td>0.27 ± 0.01†</td>
</tr>
</tbody>
</table>

Values are means ± SE from stated number of cells from 4–6 rats for normal control and treatment groups and 12 rats for septic control group. +dL/dt: maximal velocity of shortening; −dL/dt: maximal velocity of relengthening; peak ht: peak baseline height; peak t: time taken to reach peak height; t: time to 90.0% of base length. *P < 0.05 vs. septic control. †P < 0.05 vs. normal control. ‡Not significantly different from normal control. §Not significantly different from septic control.

AJP-Heart Circ Physiol • VOL 286 • MARCH 2004 • www.ajpheart.org
effect (12). ADM has been observed to produce a dose-dependent increase in developed tension in rat papillary muscle associated with an elevation in the cAMP content sensitive to suppression by PKA inhibitors, suggesting involvement of a cAMP-dependent pathway (13). ADM has also been seen to stimulate cAMP accumulation in rat cardiomyocytes up to 5.5-fold compared with 38-fold by 10 μM forskolin (39). In contrast, in rabbit ventricular myocytes, ADM had a negative inotropic effect and was observed to decrease Ca\(^{2+}\) transient and ionic current, and these effects were abolished by pretreatment with the NO synthase (NOS) inhibitor N-nitro-L-arginine. In rabbit ventricular myocytes, ADM significantly increased intracellular cGMP, but not cAMP, content (15). However, in our previous study, 1H-[1,2,4]oxadiazolo-[4,3-a]quinonoxalin-1-one, a specific inhibitor of guanylate cyclase, did not affect the ADM-mediated effect (12). Moreover, pretreatment with L-NAME was without effect on the ADM-mediated actions, suggesting that the negative inotropic effect of ADM is through an NO/cGMP-independent pathway in rat ventricular myocytes (12).

To rule out the involvement of NO in the ADM-mediated effects, the interaction with COX was studied. Indomethacin, a nonspecific COX inhibitor, abolished the positive and negative inotropic effects mediated by ADM. Pretreatment with nimesulide, a sulfonamidic compound having selectivity for COX-2, also attenuated both effects, indicating involvement of COX-2. Indomethacin and SC-236, another specific COX-2 inhibitor, also abolished the changes in [Ca\(^{2+}\)], induced by ADM. In a recent report, small, variable amounts of COX-2 mRNA and protein in control, untreated cardiomyocytes were observed, suggesting that these amounts were continually present at low levels in the controls and were increased manyfold on induction with interleukin according to their protocol (24). The presence of COX-2 in untreated myocytes was stated not to be the result of LPS contamination, as much as no inducible NOS (iNOS) mRNA or protein was observed in these control samples. Also both COX isozymes have been shown to occur constitutively in several organ systems (6). Therefore, ADM seems to be interacting with COX-2 to mediate the positive and negative effects in our myocyte samples.

To ascertain further which prostanoid(s) might be involved, we pretreated the myocytes with tranylcypromine, a prostacyclin synthase inhibitor, or SQ-29548, a thromboxane receptor antagonist. Tranylcypromine abolished both ADM effects, implying that prostacyclin plays a role as a mediator. SQ-29548, a thromboxane receptor antagonist, resulted in an increase in cAMP generation consistent with IP coupling to Goᵢ, suggesting that prostacyclin may couple to Goᵢ and Goᵣ, depending on its concentration (26). On the other hand, prostacyclin might cause agonist-induced desensitization of the IP receptor (31).

To ascertain whether reversal of the long-term exposure effects to ADM and the cardiodepression observed as a result in normal cardiomyocytes could also be observed in the LPS-treated myocytes, we used a rodent septic shock model. A significant increase in levels of ADM was observed in plasma, ventricles, and isolated ventricular myocytes 4 h after the injection of bacterial endotoxin (LPS) in our present study and as described previously (20, 25). Moreover, we have observed that ADM-(22–52) was unable to improve the β-adrenergic hyporesponsiveness in shocked ventricular myocytes (data not shown). Shan and Bourreau (40) previously reported that the NOS inhibitor restores β-adrenergic hyporesponsiveness in shocked myocytes but does not restore the basal response to electrical field stimulation. In LPS-treated cells, L-NAME did not improve the cardiomyocyte contractility, ruling out the involvement of iNOS, as has been observed previously (12). The increase in Ca\(^{2+}\) transients and cell contractility by indomethacin, SC-236, and tranylcypromine in our study implies that a COX mediator causes the negative inotropy and the decrease in [Ca\(^{2+}\)] i in septic shock.

Ventricular myocytes from LPS-treated rats show decreased rates of ventricular contraction and relaxation (36). We also observed a marked decrease in the maximal velocity of shortening and in peak height and maximal velocity of lengthening and an increase in the time to peak and to relengthen to the baseline level, showing a depressed contraction and a slow relaxation by the cell compared with normal cells. In these LPS-treated cells, indomethacin markedly improved the cell contractility and restored it to near-normal levels, implying that COX has a role in LPS-induced cardiodepression. This restoration was not only in terms of cell shortening, but the time...
taken to return to base length was also significantly decreased, showing overall improvement in the cell kinetics. SC-236, a highly specific inhibitor of COX-2, also significantly improved the cell contractility with respect to cell shortening, but it did not have an effect on time taken to return to base length, which remained as depressed as in the LPS-treated cells. This difference in cell kinetics, despite the restored cell contractility by indomethacin and SC-236, indicates that not only COX-2, but also COX-1, might be involved. Furthermore, pretreatment with tranlycrompromine also restored the cell shortening, but the time taken to return to base length remained depressed, as seen in LPS-treated cells. Thus prostacyclin inhibition could restore the cell contraction but could not improve the kinetics with respect to the cell relaxation. However, SQ-29548 had no significant effect on cell shortening and improved the time to peak and the time taken to return to base length, indicating again that not only prostacyclin, but also thromboxane, might be involved in LPS-induced depression in myocyte contractility. As early as 1983, it was observed that indomethacin suppressed a cardiodepressant factor released by endotoxin in the rat, and the possible involvement of a prostacyclin-related substance was stressed (3). However, the finding that treatment with indomethacin did not completely restore the cell shortening kinetics to a level comparable to normal controls implies that factors other than involvement of COX also play a role in LPS-induced cardiodepression.

Recently, significant expression of COX-2 has been demonstrated in human septic heart and endotoxic rat heart (28, 8). Progressive liberation of prostacyclin and thromboxane has been reported to be induced by LPS, together with myocardial COX-2 mRNA expression (8). Prostacyclin synthesis is tightly coupled to expression of COX-2, whereas prostaglandin E2 and thromboxane synthesis is induced by COX-1 or COX-2, depending on the stimulus (47). No role of cardiac NO has been observed to contribute to the negative inotropism of LPS (8). Plasma levels of cytokines such as TNF-α and IL-1 have been observed to markedly increase in experimental endotoxemia as well as in septic patients (8, 22). ADM secretion and its mRNA levels in cardiomyocytes have been seen to increase significantly after stimulation with IL-1β; also, ADM gene expression could be augmented by TNF-α (10, 42). Because the above-mentioned mediators increase earlier than the increase in circulating levels of ADM, as well as the onset of the early hyperdynamic phase, it appears that these cytokines are responsible for upregulating ADM after the onset of sepsis, and then ADM acts as a compensatory mediator in the early phase of septic shock. A recent study suggests that LPS and IL-1β activate the endothelial PAS domain protein (EPAS)-1 at the transcriptional level, which in turn induces ADM gene expression in rat cardiomyocytes (43). Administration of synthetic rat ADM in normal animals, at a dose that does not significantly reduce blood pressure, produced the hyperdynamic and hypercardiovascular response similar to that observed during the early stage of sepsis (46). Moreover, administration of anti-ADM antibodies, early after the onset of sepsis, prevented the increase in cardiac output, heart performance, and tissue perfusion (46). Our results also show ADM as a positive inotropic agent if incubated for a short time in normal cardiomyocytes. A striking observation in this study has been the reversal of depressed myocardite contractility in LPS-treated cardiomyocytes, isolated during the late hypodynamic stage of septic shock, on pretreatment of the samples with anti-ADM (rat) IgG. “In vitro” treatment with 300 nM ADM antiserum in a small sample of myocytes completely reversed the contractility to normal levels when observed within 1 h. With treatment with the ADM antiserum for >1 h, the effect was observed to wane, and within 2 h it was completely lost (data not shown), implying that ADM might be generated continuously in LPS-treated myocytes, such that the effect of ADM antiserum was saturated with time.

If we take all the results together, it seems plausible that, on progression of the early hyperdynamic stage of septic shock into the late hypodynamic stage, the role of ADM reverses from that of a compensatory mediator to a perpetrator of cardio depression. ADM seems to be mediating its effects mainly through activation of COX-2, because its specific inhibitor could reverse the increased and decreased cardiomyocyte contractility and changes in [Ca2+]i in normal and LPS-treated cells. COX-2-induced production of prostacyclin may be mediating the positive or negative inotropic effects of ADM and the accompanying changes in [Ca2+]i, implying that the absolute level of cAMP induced may be a crucial determinant of the cellular contractility.

GRANTS

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ROLE OF ADRENOMEDULLIN IN LPS-INDUCED SEPTIC SHOCK


