Differential Effects of Amrinone and Milrinone Upon Myocardial Inflammatory Signaling

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Background—Mounting evidence links systemic and local inflammatory cytokine production to myocardial dysfunction and injury occurring during ischemia-reperfusion, cardiopulmonary bypass, and heart failure. Phosphodiesterase inhibitors (PDEIs), used frequently in these states, can modulate inflammatory signaling. The mechanisms for these effects are unclear. We therefore examined the effects of 2 commonly used PDEIs, amrinone and milrinone, on cardiac cell inflammatory responses.

Methods and Results—Primary rat cardiomyocyte cultures were treated with endotoxin (LPS) or tumor necrosis factor-α (TNF-α), alone or in the presence of clinically relevant concentrations of amrinone or milrinone. Regulation of nuclear factor-kappa B (NFκB), nitric oxide synthase and cyclooxygenase isoforms, and cytokine production were assessed by electrophoretic mobility shift assays, Western immunoblotting, and enzyme-linked immunoassays, respectively. Both LPS and TNF-α induced significant NFκB activation, cyclooxygenase-2 (COX-2) expression, and inducible NO synthase (iNOS) and cytokine production; with the exception of COX-2 expression, all were significantly reduced by amrinone, beginning at concentrations of 10 to 50 μmol/L. In contrast, milrinone increased nuclear NFκB translocation, iNOS and COX-2 expression, and cardiomyocyte production of interleukin-1β. Cell-permeable cAMP increased inflammatory gene expression, whereas cell-permeable cGMP had no effect, indicating that the effects of amrinone were not due to phosphodiesterase inhibition. Similar results were seen in macrophages and coronary vascular endothelial cells.

Conclusions—Both amrinone and milrinone have significant effects on cardiac inflammatory signaling. Overall, amrinone reduces activation of the key transcription factor NFκB and limits the production of pro-inflammatory cytokines, whereas milrinone does not. (Circulation. 2002;106[suppl I]:I-284-I-289.)

Key Words: inflammation • nitric oxide synthase • inotropic agents • cytokines • phosphodiesterase inhibitor • cyclooxygenase

Stimulation of local and systemic inflammatory mediator production occurs as a consequence of cardiopulmonary bypass (CPB), major surgery and tissue trauma, sepsis, and ischemia-reperfusion. These responses include activation of a wide range of interrelated pathways including: endotoxin release and cytokine production; white blood cell and endothelial cell activation and expression of leukocyte adhesion molecules; production of nitric oxide, oxygen radicals, and proteolytic enzymes; complement, coagulation, fibrinolytic, and kallikrein systems.1-3 The efficacy of specific therapies directed against these pathways (eg, monoclonal antibodies, receptor or enzyme blockade) in clinical or experimental models of CPB and inflammation has been modest, at least in part because of the diverse and interwoven nature of the pathways involved. More general therapies, such as steroids, have well-known negative effects.

Phosphodiesterase inhibitors (PDEIs), such as amrinone and milrinone, are frequently used for their inotropic, lusitropic, and vasodilating properties; an oral PDEI, vesnarinone, has been used to treat heart failure. Recently, numerous and incompletely characterized immunomodulating properties have been attributed to various members of this class of compounds. These effects included decreased pro-inflammatory cytokine production in humans and experimental models, and decreased myocardial inflammation and T cell activity in experimental myocarditis.4-7 Increased circulating and myocardial cytokine concentrations have also been linked to human heart failure; this finding, together with those mentioned, has led to speculation that the antiinflammatory effects of these compounds may be responsible for some of the clinical improvements resulting from their use.8

We have previously found that these agents may reduce markers of systemic inflammation and improve myocardial...
function and calcium handling in animal endotoxemia models. On the other hand, increased cellular concentrations of cAMP, which would be expected to result from PDE inhibition, may theoretically increase inflammatory stimuli via binding to cAMP response elements present in some proinflammatory genes (eg, iNOS, COX-2). To better understand the mechanisms and limitations of potential antiinflammatory effects of PDEIs in the heart, we exposed cardiomyocytes, coronary endothelial cells, and macrophages to various inflammatory stimuli. We examined the effects of 2 commonly used PDEIs, amrinone and milrinone, on key readouts of inflammatory stimulation. These included activation of the transcription factor NFκB, production of TNF and IL-1, induction of iNOS and COX-2 expression; on the vascular endothelial cells, we also measured expression of the leukocyte adhesion molecule intercellular adhesion molecule 1 (ICAM-1).

Methods

Cell Culture and Treatments

Two-day-old Wistar rat (Charles River Laboratories) cardiomyocytes were isolated as described earlier using the Neonatal Cardiomyocyte Isolation System (Worthington). Contaminating cardiac fibroblasts were removed by pre-plating cell mixtures for 1.75 hours in 75-mm² culture flasks (Becton Dickinson). Cardiomyocytes were initially plated in DMEM-F12 (Life Technologies) containing 10% fetal bovine serum (FBS), 10 μM/μL cytosine β-D-arabinofuranoside (Sigma), 1% penicillin/streptomycin (Life Technologies), and allowed to attach for 24 hours. Cells were subsequently maintained in a defined media of DMEM-F12 containing 10 μM/μL cytosine β-D-arabinofuranoside, 1% insulin, transferrin, and selenium solution (ITS; Collaborative Biomedical), 0.25 mg/mL fetuin (Sigma), 0.1X MEM vitamins (Life Technologies), 0.4X nonessential amino acids, 1% penicillin/streptomycin, 15 mmol/L NaHCO₃, and 0.5 mmol/L CaCl₂.

The RAW264.7 mouse macrophage cell line was purchased from Clonetics (BioWhittaker) cultured according to the manufacturer directions in specified defined media, and used at passage 3 or 4. Cell cultures were treated for 24 hours with 1.0 μg/mL Salmonella typhosa LPS (Sigma) alone or in combination with 10, 50, 100, or 250 μg/mL amrinone or milrinone. Other cultures were exposed only to 250 μg/mL amrinone or milrinone in the absence of LPS to control for the effects of these inhibitors.

Immunoblot Analyses

Proteins were isolated by rinsing the cells 2 times with ice-cold phosphate-buffered saline (pH 7.4), then lifting them from the plates with a rubber policeman. Cells were removed to 1.5-mL tubes and collected with a 10-second spin at 12 000 g. Cells were resuspended in a small volume of ice-cold lysis solution (150 mmol/L NaCl, 20 mmol/L Tris-HCl pH 7.6, 1 mmol/L EDTA, 0.5% sodium deoxycholate, 70 mmol/L NaF, 1% Nonidet P-40, Complete protease inhibitor cocktail [Boehringer Mannheim], 200 μmol/L sodium orthovanadate, and 2 μmol/L phenylmethylsulfonyl fluoride). After a 10-minute incubation on ice with intermittent, brief vortexing, debris was pelleted in the microcentrifuge and the supernatants were stored at −80°C. Protein concentrations were established using the Pierce BCA protein determination kit.

SDS-PAGE and transfer to nitrocellulose was performed as described earlier and identical gels were stained with Coomassie brilliant blue R250 to confirm equal protein loading. Nitrocellulose membranes were rinsed in Tris-buffered saline pH 7.4 containing 0.1% Tween-20 (TBS-T) and blocked in 5% nonfat milk; TBS-T for 1 hour at 22°C on a rocking platform. Membranes were rinsed 4 times in TBS-T then incubated overnight at 4°C on an orbital shaker with primary antibodies diluted in TBS-T (Sigma). Immunoblots were incubated with a mouse anti-COX-1 monoclonal antibody (Cayman), a rabbit anti-COX-2 polyclonal antibody (Cayman), or a polyclonal rabbit anti-iNOS antibody at the manufacturer’s suggested dilutions (1:1000). The excess primary antibodies were washed from nitrocellulose membranes with 3 10-minute washes in TBS-T and incubated with appropriate horseradish peroxidase-conjugated anti-rabbit or anti-mouse secondary antibodies (Amer sham) diluted 1:2500 in 5% milk/TBS-T. Following 3 10-minute washes in TBS-T, bound antibodies were detected with the ECL kit (Amer sham) and exposed to Kodak X-Omat AR film.

Endothelial Cell Surface Immunoassay for Intercellular Adhesion Molecule-1

Endothelial cell expression of intercellular adhesion molecule-1 (ICAM-1) was measured with a cell surface enzyme-linked immunosorbent assay (ELISA), as previously described. Briefly, endothelial cells were cultured in 96-well plates, treated as described above, fixed with ice-cold paraformaldehyde in phosphate-buffered saline, washed with Hanks’ balanced salt solution, and blocked with medium containing 5% FBS for 30 minutes at 4°C. Anti-ICAM-1 monoclonal antibody (R&D Systems) was then added at a concentration of 10 μg/mL for 2 hours at 4°C. After washings with HBSS, peroxidase-conjugated sheep anti-mouse secondary antibody (Cappel), at a 1:1000 final dilution in medium containing 10% FBS, was added, washed with HBSS, and developed by addition of peroxidase substrate (2,2’-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) and read at a wavelength of 405 nm on a microtiter plate spectrophotometer (Molecular Devices). Controls consisted medium only and secondary antibody only.

Nitrite Analysis

Nitric oxide enzyme production was estimated in culture media using the Greiss reagent as described earlier and adjusted for protein content differences between cells and treatments using the BCA assay (Pierce).

Electrophoretic Mobility Shift Assays

Nuclear extracts were isolated from treated cultures using the method of Andrews and Faller and quantitated using the BCA protein determination kit (Pierce). Electrophoretic mobility shift assay reactions were carried out as described previously. Briefly, each 20-μL reaction contained 10 mmol/L Tris-HCl pH 7.6, 70 mmol/L KCl, 5 mmol/L MgCl₂, 1 mmol/L DTT, 1 mmol/L EDTA pH 8.0, 25% glycerol, 0.2% Triton X-100, Complete protease inhibitor, 2.0 μg poly (dl-dC)·poly (dl-dC) (Pharmacia), 0.25-ng labeled NFκB, (Promega) consensus oligonucleotides, and, in some reactions, unlabelled oligonucleotides (either identical or unrelated but same size). Probes were labeled with [γ³²P]-ATP and polynucleotide kinase using standard techniques and purified with ProbeQuant G-50 columns (Pharmacia).

Cytokine Assays

TNF-a and IL-1β production were measured in cell culture media by specific ELISA according to the manufacturer’s instructions (R&D Systems).

Statistical Analyses

All data are presented as means±standard deviation (SD). Results are from at least 6 separate determinations. Densitometric determination of integrated volumes under the curve of unsaturated radiographs of immunoblots were determined using Scion Image software (NIH). Multiple group comparisons were made by analysis of variance (ANOVA) followed by the Tukey-Kramer test, except when comparing fold-changes in immunoblot band density, in which the nonparametric Kruskal-Wallis test was used.
Results

Cardiomyocytes

Both LPS and TNF-α stimulated significant activation of NFκB (Figure 1) and induction of iNOS (Figure 2) and COX-2 synthesis (Figure 3). LPS caused significant production of IL-1β and TNF-α by cardiomyocytes (Figure 4).

Based on gel shift analyses (Figure 1), the inhibitory effect of amrinone on LPS-driven NFκB activation began at approximately 50 μmol/L. When TNF was the stimulus, interestingly, amrinone appeared to increase NFκB activation up to concentrations of 50 μmol/L, but it was inhibitory at higher concentrations. Milrinone had essentially no effect on LPS-stimulated NFκB activation, and, in fact, increased that stimulated by TNF at all tested concentrations.

Amrinone suppressed LPS- and TNF-stimulated induction at concentrations of 50 and 100 μmol/L, respectively (Figure 2). In contrast, milrinone had no effect on induction of iNOS in response to LPS, and caused a dose-dependent increase in iNOS enzyme production in response to TNF. These changes in iNOS protein were closely paralleled by changes in NO production measured by Griess assay (not shown).

As opposed to the effects on iNOS, amrinone had no apparent effect on production of COX-2 (Figure 3) except at very high concentrations (≥250 μmol/L). Milrinone once again increased COX-2 production at all tested concentrations.

The effects of amrinone on LPS-stimulated production of TNF-α and IL-1β are shown in Figure 4. Interestingly, both amrinone and milrinone caused a dose-dependent decrease in
Figure 4. Cardiomyocyte TNF-α (A) and IL-1β (B) production (measured by ELISA) in response to LPS in the presence of varying concentrations of either amrinone (solid lines) or milrinone (broken lines). Results are after 24-hour exposure to 1 μg/mL LPS. X-axis denotes concentration (μM) of amrinone or milrinone. Data are means±SD, N=5 to 8 each. Reductions in LPS-stimulated TNF production for both amrinone and milrinone were significant compared with LPS alone (P<0.05) beginning at 50 μmol/L. Amrinone was significantly more potent at suppressing TNF production at 100 and 250 μmol/L. Amrinone completely suppressed IL-1 production beginning at 50 μmol/L (P<0.05 versus no amrinone).

TNF-α production. However, milrinone caused a modest increase in cardiomyocyte IL-1β production in response to LPS, whereas amrinone decreased IL-1β production.

Treatment of cardiomyocytes with exogenous rat IL-1β also led to activation of NFκB and marked production of iNOS. These effects were similarly suppressed by amrinone and increased by milrinone (not shown).

To specifically assess the role of phosphodiesterase inhibition and resultant increases in intracellular concentrations of cyclic nucleotides, treated cells were exposed to either 8-bromo-cAMP or 8-bromo-cGMP (1 to 100 μmol/L; Bicamol), which are cell-permeable analogs of the respective cyclic nucleotide. In all cell types studied, when TNF-α or IL-1β was the stimulus, 8-bromo-cAMP caused dose-dependent and significant (P<0.05) increases in iNOS and COX-2 production. When LPS was the stimulus, 8-bromo-cAMP once again increased iNOS, COX-2, and IL-1β production (P<0.05) in all cell types. The only inhibitory effect of 8-bromo-cAMP was to significantly reduce TNF production (at concentrations >25 μmol/L) in cardiomyocytes and RAW cells exposed to LPS. 8-bromo-cGMP had no effect on any of the cell types or stimuli studied.

Coronary Microvascular Endothelial Cells and Macrophages

The general applicability of the cardiomyocyte results were tested in an immune cell model (the RAW macrophage cell line) and cultured human coronary microvascular endothelial cells (HCMVECs). The effects in RAW cells are summarized in Table I. Similar to cardiomyocytes, amrinone caused substantial and dose-dependent inhibition of macrophage inflammatory responses.

ICAM-1 is a major leukocyte adhesion molecule. The effects of amrinone and milrinone on ICAM-1 expression in HCMVECs are shown in Figure 5. Amrinone significantly reduced ICAM-1 expression in response to LPS, whereas it was increased by milrinone.

Effects of Amrinone and Milrinone Upon Mediator Production in RAW Cells

<table>
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<tr>
<th>Stimulus</th>
<th>INOS</th>
<th>TNF-α</th>
<th>IL-1β</th>
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<tr>
<td></td>
<td>Amrinone</td>
<td>Milrinone</td>
<td>Amrinone</td>
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<tr>
<td>LPS, 1 μg/mL</td>
<td>16±6‡</td>
<td>128±16‡</td>
<td>26±5‡</td>
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<tr>
<td>TNF, 1 ng/mL</td>
<td>25±5‡</td>
<td>137±15‡</td>
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RAW cell macrophages were treated for 24 hours with LPS or TNF as indicated. Data represent the percent change in expression of iNOS, TNF-α, or IL-1β in the presence of 50 μM amrinone or milrinone compared to that produced by LPS or TNF alone. INOS expression was measured by immunoblotting and quantified by densitometry (see text). TNF-α and IL-1β production were measured by ELISA. Data are mean±SD from 4 separate experiments each.

†P<0.05 vs LPS or TNF alone (significant decrease); ‡P<0.05 vs LPS or TNF alone (significant increase); ††P<0.05 compared to amrinone response.
In HCMVECs, LPS significantly (7-fold, \( P<0.01 \)) increased COX-2 expression. The LPS-induced increase in COX-2 expression in HCMVECs was unaffected by either phosphodiesterase inhibitor. Endothelial cell expression of the constitutive COX-1 isoform was unaffected by either LPS or the PDEIs.

**Discussion**

Numerous studies now indicate the importance of various components of inflammation in cardiac injury and dysfunction resulting from sepsis, ischemia-reperfusion, CPB, and heart failure. Although the models used in the present study were all cell-culture based and may most directly simulate Gram-negative endotoxemia, the results are also relevant to CPB. First of all, endotoxin release can occur during CPB. Like CPB, endotoxemia activates cytokine, white cell, complement, and adhesion molecule pathways. CPB has also been associated with increased production of TNF-\( \alpha \) and other cytokines; ischemia-reperfusion rapidly stimulates myocardial TNF-\( \alpha \) production. The most significant finding of the present study was that amrinone, but not milrinone, significantly reduced the production of multiple markers of inflammatory stimulation.

The ability of amrinone to suppress pro-inflammatory cytokine production is qualitatively similar to that observed previously in a whole animal model of endotoxemia. The present study significantly expands on these results because it demonstrates that the effect of amrinone applies to multiple cell types and also to very different inflammatory stimuli (eg, LPS, TNF, IL-1) that employ distinct membrane receptors and membrane signaling complexes. This broad effect on a number of inflammatory stimuli and against a number of inflammatory gene products indicates that amrinone is likely to be acting downstream at one or more central and convergent points of inflammatory signaling. The results are also significant because they demonstrate that the anti-inflammatory effects of amrinone are largely not shared by milrinone, the other commonly used PDEI.

The mechanisms for the immunomodulating effects of PDEIs have been unclear. One obvious possibility would be inhibition of cyclic nucleotide (cAMP or cGMP) breakdown. As was seen in the present study, cAMP can block LPS-stimulated TNF production; however, this effect may be specific for LPS-stimulated TNF release, because production of other cytokines was not inhibited under similar circumstances. Overall, cAMP has been shown to both increase and inhibit pro-inflammatory signaling, depending on stimulus, cell type, and inflammatory product studied, with inhibitory effects predominating. Milrinone increased production of iNOS, COX-2, and IL-1 in cardiomyocytes, ICAM-1 in LPS-stimulated HCMVECs, and iNOS in RAW cells. These effects were mimicked by cAMP (but not cGMP). Taken together, these results suggest that milrinone-stimulated increased cAMP was responsible. Although amrinone would be expected to have similar effects on intracellular cyclic nucleotide levels, it must also have other and overriding effects on inflammatory signaling that result in net inhibition of these same signals.

The present study for the first time presents data to suggest that inhibition of NF\( \kappa \)B activation is one likely site of the anti-inflammatory effect of amrinone. NF\( \kappa \)B activation is widely acknowledged to be required for expression of a number of inflammatory and stress-response genes, including those stimulated by LPS and TNF and those resulting in TNF and iNOS production. How amrinone might be interfering with NF\( \kappa \)B activation is unclear at present. Release of membrane phosphatidic acid is a common second messenger response to a variety of pro-inflammatory and membrane-damaging stimuli that results in cellular activation; some PDEIs have been shown to inhibit phosphatidic acid production. It is unknown at present whether NF\( \kappa \)B activation is linked to release of phosphatidic acid release or if amrinone is acting via this mechanism.

It is also important to note that the effects of amrinone may not be exclusively beneficial. In addition to its ability to stimulate numerous inflammatory gene programs, NF\( \kappa \)B may also evoke cell survival and anti-apoptotic programs, as well as the myocardial protection associated with ischemic preconditioning. LPS and TNF have been shown to induce delayed preconditioning, heat shock proteins, antioxidant enzymes, and other potentially cytoprotective functions. Inhibition of iNOS-mediated NO release in an inflammatory milieu may in fact exacerbate organ damage because of the loss of the effects of NO production to maintain organ blood flow and decrease leukocyte infiltration.

In this regard, the lack of PDEI effect on COX-2 induction by pro-inflammatory stimuli is also noteworthy. The most frequently cited paradigm is that COX-1 serves homeostatic functions (eg, gastric mucosal integrity and renal blood flow), whereas COX-2 is induced at sites of inflammation and contributes to inflammatory pathogenesis (eg, arthritis). Recent evidence indicates that COX-2 products may also be cytoprotective, via mechanisms that include inhibiting transcription of pro-inflammatory cytokine genes, inhibiting apoptosis, and improving regional blood flow. Genetic disruption of COX-1 and COX-2 genes was recently associated with increased cardiac ischemia-reperfusion injury. Prostacyclin derived from COX-2 (or supplied exogenously as a stable prostacyclin analog) can limit damage to cardiomyocytes from oxyradical injury caused by hydrogen peroxide or doxorubicin. In vascular endothelial cells, which have substantial quantities of prostacyclin synthase, genetic overexpression of COX-2 increased production of prostacyclin (PGI\(_2\)). On the other hand, when COX-2 overexpression was stimulated by cytokines in human umbilical vein cells, there was increased production of PGH\(_2\) that could not be totally converted into protective PGI\(_2\). This appeared to be due to inhibition of PGI synthase by cytokine-stimulated NO or NO-derived species, leading to nonenzymatic production of PG E\(_2\) (pro-inflammatory), PGD\(_2\), and PGF\(_{2\alpha}\) (vasoconstricting). In the present study, milrinone appeared to induce this same combination of increased COX-2 and NO production, which could lead to the accumulation of deleterious prostanooids. Using the same paradigm, amrinone administration in the setting of inflammation may, by maintaining COX-2 and reducing iNOS, favor continued synthesis of...
protective PGI₂. Further experiments are required to test these questions.

In conclusion, PDEIs that are in common clinical use have significant effects on inflammatory signal transduction pathways and mediator production. Overall, amrinone appears to have substantial and potentially beneficial effects on diverse pro-inflammatory stimuli, whereas milrinone does not. These effects appear to be mediated, at least in part, by interfering with the nuclear transcription factor NFkB. A trial of amrinone, given before and during CPB, may be warranted as a means to reduce inflammatory signaling.

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References
