Mechanisms of desensitization to a PDE inhibitor (milrinone) in conscious dogs with heart failure

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Sato, Naoki, Kuniya Asai, Satoshi Okumura, Gen Takagi, Richard P. Shannon, Yoko Fujita-Yamaguchi, Yoshihiro Ishikawa, Stephen F. VATNER, and Dorothy E. VATNER. Mechanisms of desensitization to a PDE inhibitor (milrinone) in conscious dogs with heart failure. Am. J. Physiol. 276 (Heart Circ. Physiol. 45): H1699–H1705, 1999.—The goal of this study was to determine the extent to which the effects of milrinone were desensitized in heart failure (HF) and to determine the mechanisms, i.e., whether these effects could be ascribed to changes in cAMP or phosphodiesterase (PDE) activity in HF. Accordingly, we examined the effects of milrinone in seven conscious dogs before and after HF was induced by rapid ventricular pacing at 240 beats/min. The dogs were chronically instrumented for measurements of left ventricular (LV) pressure and first derivative of LV pressure (dP/dt), arterial pressure, LV internal diameter, and wall thickness. Milrinone (10 µg·kg⁻¹·min⁻¹·iv) increased LV dP/dt by 1,854 ± 157 from 2,701 ± 105 mmHg/s (P < 0.05) before HF. After HF the increase in LV dP/dt in response to milrinone was attenuated significantly (P < 0.05): it increased by 615 ± 67 from 1,550 ± 107 mmHg/s, indicating marked desensitization. Because of this limitation, other

One major limitation to catecholamine therapy in heart failure (HF) is the desensitization that develops, rendering subsequent administration of catecholamines ineffective. Because of this limitation, other inotropic agents have been developed for use in HF. One important class of inotropic agents utilizes a mechanism of phosphodiesterase (PDE) inhibition, thereby enhancing the level of cAMP without depending on the β-adrenergic signaling pathway. In part, the rationale for the utility of this class of agents is that cAMP levels are depressed in HF. However, the extent to which this has been documented remains controversial. For example, studies have found modest decreases in cAMP levels in HF (5, 10, 36) or no change (19, 24, 34). However, even studies demonstrating decreases in cAMP in patients may overestimate changes due to fibrosis (14). Furthermore, whether the action of PDE inhibitors undergoes desensitization in HF is not known, since few studies have compared the effects of these agents in the presence and absence of HF.

Accordingly, the first aim of the present investigation was to determine the extent to which the action of milrinone, a PDE inhibitor, undergoes desensitization in HF. The experiments were conducted in conscious dogs before and after HF was induced with rapid ventricular pacing (17, 25). A second goal was to determine the extent to which milrinone’s action depended on activation of the autonomic nervous system by examining its effects in the presence and absence of ganglionic blockade. After the response to milrinone was found to be desensitized in the physiological experiments, a third goal was to determine the mechanism of altered action of milrinone in HF, specifically if decreased PDE activity could account in part for the desensitized response to milrinone in HF. This was accomplished by measuring cAMP levels and PDE activity directly in control dogs and dogs with HF. Our hypothesis was that the changes in wall stresses and limited coronary reserve, which are primarily subendocardial, result in transmural differences in cAMP and PDE activities in HF. Accordingly, left ventricular (LV) endocardial and epicardial samples were measured in dogs with HF and compared with values in control animals.

METHODS

Instrumentation

Seven adult mongrel dogs of either gender weighing 20–30 kg were anesthetized with halothane (0.5–1.5 vol%) and ventilated with a Harvard respirator after induction with thiopental sodium (10–20 mg/kg iv). Sterile technique was used to perform a left thoracotomy through the fifth intercostal space. Tygon catheters (Norton Elastic and Synthetic Division, Akron, OH) were implanted in the descending thoracic aorta and left atrial appendage. A solid-state minia
ture pressure gauge (model P6, Konigsberg Instruments, Pasadena, CA) was implanted in the LV through the apex. Piezoelectric ultrasonic dimension crystals were implanted on opposing anterior and posterior endocardial surfaces of the LV to measure the LV internal diameter and on opposing endocardial and epicardial surfaces to measure wall thickness. The subendocardial wall thickness crystal was implanted obliquely to avoid damage to the myocardium between the two wall thickness crystals. Proper alignment of the paired crystals of LV internal diameter and wall thickness was obtained by positioning the crystals with the greatest amplitude and shortest transit time during surgery. A screw-in-type pacing lead was attached to the right ventricular free wall, and left atrial pacing electrodes were implanted. Catheters and leads were externalized, and the thoracotomy was closed. The chest was evacuated of air and fluid. Each dog was treated with 1 g of cephalothin for 10 days after surgery. Animals used in this study were maintained in accordance with the Guide for the Care and Use of Laboratory Animals [DHHS Publ. No. (NIH) 83-23, revised 1996, Office of Science and Health Reports, Bethesda, MD 20892].

**Experimental Measurements**

Experiments were initiated 2–3 wk after recovery from surgical instrumentation, when the animals were healthy, i.e., body temperature, blood cell count, and blood chemistries were within normal limits. Hemodynamic measurements were recorded with the dogs fully awake, lying quietly on their right side. The fluid-filled catheters in the aorta and left atrium were connected to strain-gauge manometers (Statham Instruments, Oxnard, CA) for the measurement of arterial and atrial pressures. LV pressure was measured with a solid-state miniature pressure gauge and calibrated in vivo against systolic arterial and atrial pressure measurements. LV wall thickness and LV internal diameter were measured with an ultrasonic transit-time dimension gauge (21). A cardiotachometer triggered by the pressure pulse provided with an ultrasonic transit-time dimension gauge (21). A solid-state miniature pressure gauge and calibrated in vivo and atrial pressures. LV pressure was measured with a solid-state miniature pressure gauge and calibrated in vivo against systolic arterial and atrial pressure measurements. LV wall thickness and LV internal diameter were measured with an ultrasonic transit-time dimension gauge (21). A cardiotachometer triggered by the pressure pulse provided instantaneous and continuous records of heart rate. The position of all catheters and crystals was confirmed after the animals were killed.

**Experimental Protocol**

Seven dogs were studied in the control state. The 5-min infusions of each dose of a PDE inhibitor, milrinone (2, 5, and 10 µg·kg⁻¹·min⁻¹ iv), were examined. On a separate day, 5-min infusions of milrinone were examined in the presence of ganglionic blockade with hexamethonium (30 mg/kg iv) and atropine (0.1 mg/kg iv) in seven dogs. After control experiments, rapid ventricular pacing was initiated at 240 beats/min and controlled using a programmable pacemaker (model EV4543, Pace Medical, Waltham, MA), which was worn externally in a vest. The experiments were repeated at 3–4 wk after pacing. All data were collected during normal sinus rhythm and with atrial pacing at 160 beats/min after a 30-min stabilization period following cessation of pacing.

**Biochemical Studies**

cAMP levels. Twelve sham-operated controls and nine HF dogs (4 of these dogs were also used for physiological studies) were anesthetized with pentobarbital sodium (30–50 mg/kg iv), and at the time of euthanasia, LV tissue was removed and separated into endocardial and epicardial samples immediately (<15 s). After connective tissue was trimmed away, the samples were placed in liquid nitrogen. LV cAMP levels were measured by cAMP [³²P]RIA kit (DuPont, Boston, MA) (32). LV tissue was homogenized in 1 ml of cold 6% TCA with a Polytron PT generator (Brinkmann, Westbury, NY). [³²P]cAMP (4,000 counts/min (cpm)) was added as a tracer to determine recovery. The homogenate was centrifuged at 2,500 g at 4°C for 15 min. The TCA was removed by extraction with water-saturated ether. The aqueous phase was vortexed and lyophilized. The lyophilized residue was resuspended in the RIA kit buffer, and the amount of [³²P]cAMP was then counted in a gamma counter.

PDE activity. In an additional seven sham and six HF dogs, PDE activity was measured using [³H]cAMP and [(¹²⁵)I]cAMP to evaluate the hydrolyzed cAMP level (2, 27). Myocardial tissue was homogenized using a ground-glass homogenizer in a buffer containing 10 mM TES, pH 7.0, 0.25 M sucrose, 1 mM phenylmethylsulfonyl fluoride, 1.3 mM N-acetyl-L-arginine ethyl ester, and 1 µg/ml each of leupeptin (Sigma Chemical) and pepstatin A (Sigma Chemical). The homogenate was centrifuged at 2,000 g for 5 min. The supernatant was filtered through cheesecloth, and any myocardial debris was discarded. The supernatant was recentrifuged at 100,000 g for 30 min. The 100,000-g supernatant and pellet corresponded to the cytosolic fraction and particulate fraction, respectively. The pellet was resuspended in an appropriate volume of homogenization buffer. Samples of the supernatant and particulate fractions were assayed immediately for PDE activity or stored at −80°C. Preliminary studies showed that there was no difference in PDE activity after freezing at −80°C.

The enzyme activity was measured according to the two-step assay procedure outlined in previous reports (18, 33, 40) with some modifications. The [²⁸³H]cAMP ([NEN Research Products, Boston, MA) was converted by PDE to [²⁸³H]cAMP in the first step and was subsequently converted to [²⁸³H]adenosine by addition of nucleotidase in the second step. In the first step the cytosolic or particulate fraction was incubated for 10 min in 0.1 ml of 40 mM TES buffer, pH 7.5, 4 mM MgSO₄, and 0.5 mM cAMP ([²⁸³H]cAMP, 100,000 cpm/assay) at 30°C. The reaction was stopped by heating in a heating block at 100°C for 120 s. The reaction mixture was then cooled to −20°C for 4 min, and 10 µl of Crotalus atrox snake venom (Sigma Chemical; 5 mg/ml) were added. The second step reaction was allowed to proceed for 20 min at 37°C. Unreacted cAMP was removed by mixing with 1 ml of a 33% slurry of Dowex AG-1X2 (Bio-Rad, Richmond, CA). In a preliminary study we confirmed that the assay was linear up to 15 min of incubation time and 15 µg/assay of the protein used in an assay. The mixture was rocked at 4°C for 5 min and centrifuged at 2,200 g. A 0.5-ml aliquot of the supernatant was counted in a liquid scintillation counter. PDE activity was expressed as picomoles of cAMP hydrolyzed per minute per milligram of protein. The protein used in this assay (8 µg/assay) was determined by the method of Bradford (6) with BSA as a standard. The mean of the interassay coefficient of variation was 0.06 ± 0.02 in sham-operated controls and 0.07 ± 0.01 in the dogs with HF, and the mean of the intra-assay coefficient of variation was 0.02 ± 0.01 in sham-operated controls and 0.02 ± 0.01 in the dogs with HF.

**Data Analysis**

Hemodynamic measurements were recorded on a multichannel tape recorder (Honeywell, Denver, CO) and played back on a direct-writing oscillograph (Gould, Cleveland, OH). LV pressure, internal diameter, and wall thickness analog signals were digitized (500 Hz) and analyzed using a computer-based system (Notocord, Croissy, France). LV end diastole was defined as the point of the beginning of positive LV dp/dt. LV end systole was defined as the point of the peak negative LV dp/dt. LV wall thickening was calculated as (ESW –
Effects of Milrinone on LV Function Before and After HF in the Presence of Ganglionic Blockade

In the presence of ganglionic blockade, milrinone still increased heart rate significantly before HF: +17 ± 2 beats/min (P < 0.05; Fig. 3). This response was significantly less (P < 0.05) than in the absence of ganglionic blockade. The tachycardia response to milrinone was markedly diminished after HF: +4 ± 2 beats/min (P < 0.05 vs. before HF). The responses of LV dP/dt to milrinone were also significantly (P < 0.05) decreased (+445 ± 65 mmHg/s) before HF compared with responses in the absence of ganglionic blockade (+1,854 ± 157 mmHg/s). The response of LV dP/dt decreased significantly further (P < 0.05) after HF: +240 ± 65 mmHg/s.

cAMP Levels and PDE Activity

cAMP in LV endocardium was less (P < 0.05) in the dogs with HF (n = 9, 1.21 ± 0.08 pmol/mg) than in sham-operated controls (n = 12, 1.64 ± 0.16 pmol/mg), but no differences were observed in LV epicardium (Fig. 4). The average transmural decrease was 16%.

We compared PDE activity in the cytolic and particulate fractions of endocardium and epicardium between dogs with HF and sham-operated control dogs. The PDE activity of the particulate fraction of endocardium was lower in HF than in controls: 50.6 ± 12.5 pmol·mg⁻¹·min⁻¹ (n = 4) and 61.4 ± 5.5 pmol·mg⁻¹·min⁻¹ (n = 7), respectively (P < 0.05; Fig. 4). However, there were no significant

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**Table 1. Effects of milrinone on LV and systemic hemodynamics before and after heart failure**

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Baseline</th>
<th>Change from baseline</th>
<th>Heart Failure</th>
<th>Baseline</th>
<th>Change from baseline</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart rate, beats/min</td>
<td>98 ± 3</td>
<td>+26 ± 4*</td>
<td></td>
<td>128 ± 5†</td>
<td>+1 ± 1†</td>
<td></td>
</tr>
<tr>
<td>LV systolic pressure, mmHg</td>
<td>117 ± 7</td>
<td>+4 ± 4</td>
<td></td>
<td>98 ± 5‡</td>
<td>+3 ± 3‡</td>
<td></td>
</tr>
<tr>
<td>Mean arterial pressure, mmHg</td>
<td>98 ± 4</td>
<td>−6 ± 1*</td>
<td></td>
<td>82 ± 4†</td>
<td>−1 ± 3</td>
<td></td>
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<tr>
<td>LV end-diastolic pressure, mmHg</td>
<td>7.8 ± 0.8</td>
<td>−4.4 ± 0.7*</td>
<td></td>
<td>23.9 ± 1.5†</td>
<td>−5.6 ± 1.4*</td>
<td></td>
</tr>
<tr>
<td>LV dP/dt, mmHg/s</td>
<td>2,701 ± 105</td>
<td>+1,854 ± 157*</td>
<td></td>
<td>1,550 ± 107†</td>
<td>+615 ± 67†</td>
<td></td>
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<tr>
<td>V̇o2, ml·kg⁻¹·min⁻¹</td>
<td>0.79 ± 0.15</td>
<td>+0.27 ± 0.02*</td>
<td></td>
<td>0.43 ± 0.06†</td>
<td>+0.09 ± 0.02†</td>
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<tr>
<td>Fractional shortening, %</td>
<td>7.3 ± 3.0</td>
<td>+5.2 ± 0.8*</td>
<td></td>
<td>10.3 ± 1.3†</td>
<td>+1.9 ± 0.4†</td>
<td></td>
</tr>
<tr>
<td>Wall thickening, mm</td>
<td>2.3 ± 0.4</td>
<td>+0.9 ± 0.1*</td>
<td></td>
<td>1.6 ± 0.4</td>
<td>+0.3 ± 0.2†</td>
<td></td>
</tr>
<tr>
<td>Wall stress, g/cm²</td>
<td>41.0 ± 1.5</td>
<td>−1.9 ± 0.5*</td>
<td></td>
<td>44.2 ± 2.5†</td>
<td>−0.1 ± 0.2†</td>
<td></td>
</tr>
<tr>
<td>LV end-diastolic diameter, mm</td>
<td>15.0 ± 1.7</td>
<td>−8.9 ± 1.4*</td>
<td></td>
<td>53.7 ± 6.8†</td>
<td>−14.0 ± 3.3*</td>
<td></td>
</tr>
<tr>
<td>End systolic</td>
<td>98.7 ± 10.0</td>
<td>−29.1 ± 4.4*</td>
<td></td>
<td>114.2 ± 9.2</td>
<td>−18.2 ± 3.2*</td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 7. Milrinone was administered at 10 µg·kg⁻¹·min⁻¹. LV, left ventricular; dP/dt, 1st derivative of pressure; V̇o2, mean velocity of circumferential fiber shortening corrected by heart rate. *P < 0.05 change from baseline. †P < 0.05 vs. control.
changes in the cytosolic fraction of endocardium: 161.8 ± 10.8 (n = 6) and 142.3 ± 5.8 pmol·mg⁻¹·min⁻¹ in HF and sham-operated controls, respectively (n = 7).

In contrast, there were no significant changes in the cytosolic and particulate fractions of epicardium between HF and control: 151.1 ± 8.0 and 60.4 ± 4.0 pmol·mg⁻¹·min⁻¹ (n = 6) in HF cytosolic and particulate fractions, respectively, and 166.1 ± 8.5 and 62.7 ± 5.0 pmol·mg⁻¹·min⁻¹ (n = 7) in sham-operated cytosolic and particulate fractions, respectively. The average transmural decrease was 10% in the cytosolic fraction and 11% in the particulate fraction.

DISCUSSION

The bipyridine derivatives, e.g., milrinone and amrinone, represent one class of PDE inhibitors, and the basic mechanism of action of bipyridine derivatives is
to reduce the rate of breakdown of cAMP by inhibiting PDE activity, resulting in increased calcium uptake by the sarcoplasmic reticulum (1, 11, 31, 37). On the other hand, the effects of these agents also depend indirectly on the β-adrenergic receptor-adenylyl cyclase pathway, since this pathway is responsible for producing cAMP. Therefore, it might be predicted that responses to milrinone would be expected to be depressed in HF on the basis that cAMP levels were depressed (4, 12) due to downregulation of β₁-adrenergic receptors, decreases in adenylyl cyclase activity (8, 16), and β-adrenergic receptor-adenylyl cyclase coupling (16, 35). Although prior studies have found that the responses to bipyridine derivatives were lower in failing myocardium (4, 12), the mechanism has been thought to be an abnormality in cAMP production.

The studies that have measured cAMP levels in HF have not consistently found significant decreases. In fact, whether the levels of cAMP are decreased in failing myocardium is controversial. Some investigators have demonstrated decreases in cAMP levels in failing myocardium ranging from 20% to 60% (5, 10, 19, 36). However, others have demonstrated no change in cAMP levels in HF (13, 24, 34). Interestingly, in many of the studies, reduction of cAMP was based on explanted myocardium (5, 10, 19, 36) as opposed to rapidly frozen biopsies, which generally show preserved cAMP levels (24, 34).

If cAMP was diminished significantly in HF, it could explain the reduced milrinone responsiveness in HF. However, in the present investigation, although cAMP levels were diminished significantly in HF, this decrease was significant only in the subendocardium (−26%). This transmural decrease was −16% and not statistically significant. Had subepicardial biopsies been taken, no difference in cAMP would have been observed. In addition, tissue measurements of cAMP in patients with HF could be contaminated by the presence of fibrosis. This complicating factor is not important in the model of pacing-induced HF we used, since prior studies in our laboratory showed minimal increases in fibrosis with this paradigm and time course of pacing (17). Nonetheless, it is important to recognize that the quantitative reduction in cAMP levels (16%, transmurally) was significantly less than the reduction in inotropic responsiveness to milrinone (−67%). The discrepancy between the decrease in cAMP levels, on the one hand, and inotropic response to milrinone, on the other, makes this mechanism very unlikely as the cause of desensitization to milrinone in HF. It is for this reason that alterations in cAMP degradation, e.g., PDE activity, must be considered. Indeed, we observed an 18% reduction in PDE activity in the subendocardium in HF. Therefore, we can speculate that the PDE helps maintain cAMP levels in HF. The extent to which a change in PDE activity in HF was found in previous studies is controversial. Some studies have shown that PDE activity was decreased (26, 28) or not changed (3, 20, 29, 30) in human failing heart and animal models with HF. At least seven distinct mammalian PDE
families have been identified on the basis of molecular cloning and enzymatic characterization, and PDE iso-
zymes have different affinity for cAMP. The cardiotonic effects of the PDE inhibitors are suggested to be mainly
due to inhibition of the PDE III isoform (7, 9, 39). PDE
III exhibits a high affinity ("low Michaelis-Menten
constant") for cAMP. Thus we measured the PDE
activities under low cAMP concentrations (0.5 µM). The
specific activity of PDE we report here is similar to that
of rat myocardium assayed at low cAMP concentration
previously reported by Picq et al. (22, 23). The subcellu-
lar distribution of the cardiac PDE III exhibits species
variation and appears to be predominantly in the
cytosolic fraction in guinea pig, hamster, and rat (38, 39).
In contrast, canine LV muscle contains two func-
tional subclasses of PDE III: an imazodan-sensitive
form, which is membrane bound, and an imazodan-
sensitive form, which is soluble (39). Our experimen-
tal results in the present study indicate that the mecha-
nism of desensitization to milrinone is due in part
to the decrease in membrane-bound PDE III
activity, the isoform that plays an important role in
mediating cardiac contractility, in the subendocardium,
but not in the subepicardium. One alternative explana-
tion is that there is a PDE isoform switch in heart
failure, rendering the PDE isoform in HF less sensitive
to milrinone. Another alternative is that the experi-
ments are complicated by vascular PDE activity.
Although PDE activity exists in vascular wall tissue, it is
unlikely that the PDE activity measured in the heart
homogenates was affected by vascular PDE ac-

The studies with ganglionic blockade demonstrated
that a significant fraction of the desensitization to
milrinone in HF is autonamically mediated. Indeed, in
control dogs without HF, the increase in LV dp/dt in
response to the highest dose of milrinone was reduced
from +1,854 ± 157 to +445 ± 65 mmHg/s in the
presence of ganglionic blockade, which is even greater
than the reduction in the LV dp/dt response with HF in
the absence of ganglionic blockade: +1,854 ± 157
+615 ± 67 mmHg/s. Nonetheless, even in the presence
of ganglionic blockade, desensitization was still ob-
served in HF, i.e., LV dp/dt fell even further: +445 ± 65
to +240 ± 65 mmHg/s. The residual decreases in in-
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tropic response in HF in the presence of ganglionic
blockade could be explained in part by the decrease in
cAMP and PDE activity. However, it is not known
whether cAMP and PDE levels are affected similarly in
the presence of ganglionic blockade. Furthermore,
the results of the present study cannot exclude the possi-
bility that some other signaling mechanism or ion channel
defect also played a role in the desensitization.

The desensitization of the inotropic response to milri-
none in HF cannot be ascribed to differences in heart
rate. First, the increases in heart rate with milrinone
before HF were modest at best: +26 ± 4 beats/min.
Second, experiments with heart rate held constant
demonstrated similar desensitization to milrinone in
HF, as occurred when heart rate was allowed to vary.

There were two components to the heart rate response
to milrinone: one autonomic dependent and the other,
which persisted after ganglionic blockade, autonomic
independent. Both mechanisms were desensitized
with HF.

In summary, the current investigation demonstrated
marked desensitization to milrinone in the same
animals studied before and after HF. The mechanism of
desensitization to milrinone is, in part, autonamically
mediated. A component of the mechanism also involves
decreases in PDE activity and cAMP levels in the
failing heart. Importantly, these modest decreases in
cAMP and PDE activity were relegated exclusively to
the subendocardium. We previously demonstrated re-
duced subendocardial coronary reserve in this model
(25). Accordingly, it is possible that the decreases in
subendocardial cAMP and PDE were mediated by
increases in subendocardial wall stress and decreases
in subendocardial coronary reserve rather than due to
a primary defect intrinsic to failing myocardium.

This study was supported in part by National Institutes of Health
Grants HL-59139, HL-33107, HL-37404, and AG-4121.
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Received 4/1 july 1998; accepted in final form 26/January 1999.

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