Furosemide Elicits Nonuniform Reflex Responses Via Cardiac Sympathetic Afferents

Jørgen S. Petersen, Gerald F. DiBona

Abstract
To examine whether furosemide elicits a cardio-renal reflex response via stimulation of cardiac afferents, furosemide was administered intrapericardially in sinoaortic denervated rats. The role of vagal afferents was evaluated by intrapericardial (IPC) administration of furosemide before and after bilateral vagotomy. The role of cardiac sympathetic afferents was examined by IPC administration of furosemide before and after IPC lidocaine blockade in rats with bilateral vagotomy. Low-dose furosemide (10 μg) elicited renal sympathoinhibition, whereas high-dose furosemide (1000 μg) produced a rapid and transient change in efferent renal sympathetic nerve activity of either inhibitory (19/49, or 39%) or excitatory (30/49, or 61%) nature. The responses were not affected by vagotomy but were abolished by IPC lidocaine blockade. In rats with a renal sympathoinhibitory response to IPC administration of 1000 μg furosemide, both the hypotensive and sympathoinhibitory responses were inhibited by indomethacin, whereas indomethacin did not affect reflex responses in rats showing a renal sympathoexcitatory response to IPC injection of 1000 μg furosemide. We conclude that furosemide elicits a nonuniform reflex response mediated via cardiac sympathetic afferents of which the renal sympathoinhibitory response is dependent on intact cyclooxygenase function.

Key Words • furosemide • serotonin • indomethacin • blood pressure • pressoreceptors • vagotomy

Methods
Animal Preparation
Male Sprague-Dawley rats (weight, 320±6 g) were anesthetized with pentobarbital sodium (30 mg/kg IP) and placed on a heated micropuncture table maintaining rectal temperature at 37°C to 38°C. An endotracheal tube was inserted, and the animal was paralyzed with pancuronium (1 mg/kg IV, supplemented with 0.5 mg/kg IV every 30 minutes) while artificially ventilated with room air. Tidal volume and respiratory rate were adjusted to maintain arterial pH between 7.35 and 7.45 throughout the experiment.

Medical grade Tygon catheters were inserted into a femoral artery and vein for measurement of arterial pressure and intravenous infusion, respectively, and into the right jugular vein for intravenous administration of drugs. Arterial pressure was measured by a Statham P23XL pressure transducer and displayed continuously on a Grass model 7E polygraph. Heart rate (HR) was recorded by a linear cardiotachometer (Grass model 7P4) triggered by the arterial pressure waveform. Isotonic saline (3 mL/h) with pentobarbital (5 to 10 mg/kg per hour) was infused continuously into the femoral vein catheter. IPC catheters were made according to the technique of Veelken et al. Briefly, a loop of silicone tubing (inner diameter, 0.02 inch; outer diameter, 0.037 inch; Dow Corning) was separated into an inlet and an outlet arm by a small plug of silicone. Five or six small holes were made in the inlet arm, and one larger drainage hole was made in the outlet arm. The dead-space volume of the inlet arm was adjusted to 10 μL. A parasternal thoracotomy was performed between the second and the fourth ribs. The lobes of the thymus were carefully separated to expose a small portion of the roof of the pericardial sac adherent to the thymus. The roof of the pericardial sac was closed by apposing the two lobes of the thymus with polyacrylic glue. The thorax was closed in layers. An isotonic saline-filled extension catheter was coupled to the outlet arm of the pericardial sac catheter and lowered 1 m below the level of the pericardial sac. This maneuver generated a constant negative pressure in the pericardial sac.
pressure that ensured rapid drainage of injected fluid from the pericardial sac. Only preparations with rapid complete recovery of injected fluid were used in this study. At the end of the experiment, 100 µL of methylene blue–colored isotonic saline was injected into the pericardial sac, and leakage from the pericardial sac was assessed visually at autopsy. No rats had signs of leakage from the pericardial sac. In all protocols, the IPC injection volume was 100 µL, and a period of 15 to 20 minutes was allowed for drainage and recovery between IPC injections.

The left kidney was exposed via a retroperitoneal approach through a left flank incision. With a dissection microscope (×25), a branch of renal nerve from the aorticorenal ganglion was carefully isolated, and a bipolar platinum electrode was hooked on the renal nerve branch. The renal nerve activity was led through a Grass model HIP511 high-impedance probe and amplified (×3000 to 20,000) and filtered (30 to 3000 Hz) with a Grass model P511 bandpass amplifier (Grass Instruments). The amplified and filtered signal was led to an oscilloscope (Tektronix 5113) for visual representation, to an audio amplifier/loudspeaker (Grass model A48 audio monitor) for auditory representation, and to a rectifying voltage integrator (Grass model 7P10). On establishment of an optimal recording, the recording electrodes were fixed to the renal nerve branch with a silicone adhesive (Wacker Sil-Gel 604, Wacker-Chemie). To eliminate apparent renal nerve activity from the recorded signal, the renal nerve bundle was cut peripherally.

In all animals, the sinoaortic baroreceptors were denervated by bilateral cutting of the aortic depressor nerve, the superior laryngeal nerve, the pharyngeal nerve, the superior cervical ganglion, and the carotid sinus nerves.6 The effectiveness of SAD was confirmed by abolition of the bradycardia and sympathoinhibitory response to phenylephrine (0.25 µg IV).3,4

In rats with bilateral cervical vagotomy, effective bilateral vagotomy was confirmed by abolition of the sympathoinhibitory response to 2-methyl-5-HT (50 µg/kg IV) compared with the response before vagotomy.3,4

At the end of the experiment, the animals were killed by an overdose of pentobarbital, and effluent renal sympathetic nerve activity (ERSNA) was continuously recorded for a further 30 minutes as a measure of background signal.

All experimental procedures were in accordance with the University of Iowa and the National Institutes of Health guidelines for animal research.

Experimental Protocol

After surgery, the animals were allowed to stabilize for 30 to 60 minutes before the start of the experiment. Experiments were performed according to the following experimental protocols.

Dose Response

To evaluate the dose-response relation of reflex responses elicited by IPC injection of furosemide, IPC furosemide was administered in 10 rats with SAD and intact vagi in the following doses: 0 (isotonic saline), 0.1, 10, and 1000 µg. The 1000-µg dose had a pH of 6.8 and an osmolality of 330 mOsm/kg.

Vagotomy

The role of the vagal nerves in the reflex responses to IPC furosemide was evaluated by injecting 1000 µg furosemide IPC before and after bilateral vagotomy in eight rats with SAD. In addition, 200 µg 2-methyl-5-HT was given IPC in six rats before and after bilateral vagotomy.

Lidocaine

The effects of lidocaine on reflex responses elicited by furosemide and 2-methyl-5-HT were evaluated by injecting 1000 µg furosemide (n=8) or 200 µg 2-methyl-5-HT (n=5) IPC in rats with SAD and intact vagi before and after IPC blockade with lidocaine 2% wt/vol in isotonic saline. The pericardium was filled with lidocaine for 3 minutes with the outlet arm of the pericardial sac catheter closed. Then the outlet arm was opened, and fluid was rapidly drained with negative pressure applied to the outlet arm tubing via a 10-mL syringe. One minute later, the second IPC injection of furosemide or 2-methyl-5-HT was performed.

Time Control

In 10 animals with SAD and bilateral vagotomy, 1000 µg furosemide IPC was given before and after IPC administration of isotonic saline. As in the lidocaine experiments, isotonic saline was allowed to equilibrate for 3 minutes before the outlet arm was opened, and 1 minute later, the second IPC injection of furosemide was performed.

Indomethacin

In 13 rats with SAD and bilateral vagotomy, 1000 µg furosemide was injected IPC before and after IPC administration of indomethacin 10 mg/mL in isotonic saline. Indomethacin was allowed to equilibrate for 3 minutes before the outlet arm was opened, and 1 minute later, the second IPC injection of furosemide was performed.

Drugs

Pentobarbital (Nembutal sodium solution, 50 mg/mL; Abbott Laboratories) and indomethacin (indomethacin sodium trihydrate, Merck, Sharpe and Dohme) were diluted in isotonic saline on the day of experiment. Pancuronium (pancuronium bromide injection, 1 mg/mL; Astra Pharmaceutical Products, Inc) and lidocaine (lidocaine HCl, 20 mg/mL; Abbott Laboratories) were dissolved in isotonic saline and stored at room temperature. Phenylephrine (phenylephrine HCl, 10 mg/mL; Elkins-Sinn, Inc) was diluted in isotonic saline (2.5 mg/mL) and stored at 5°C. 2-Methyl-5-HT (2-methyl-5-HT maleate, Research Biochemicals, Inc) was dissolved in isotonic saline (50 mg/L) and stored at −20°C. Furosemide (furosemide injection, 10 mg/mL; Abbott Laboratories) was supplied in 10-mL vials dissolved in isotonic saline.

Data Analysis

Data were sampled on-line via a pulse code modulation recording adaptor (model 4000, AR Vetter Co) and stored on videotape with a standard videocassette recorder (Fisher model FVH-6200). Data analysis was performed off-line with an analog-to-digital converter (model DT2801, Data Translation Inc), appropriate software (Labtech Notebook version 4.2, Laboratory Technologies Corp), and an IBM PS 2 computer. Integrated ERSNA was analyzed as mean integrated voltage: (µV·s) per second. The postmortem background signal was subtracted from all nerve activity measurements. When a clear renal sympathoexcitatory or sympathoinhibitory response was observed, the peak response was defined as the average response during the 3-second period with maximal deflection from baseline. When no well-defined response was observed (after IPC isotonic saline and indomethacin), the reading was performed during the 3-second period between the 8th and 10th seconds after IPC injection. Overall statistical analysis was performed with one-way analysis of variance (ANOVA). Student’s paired or unpaired t test with Bonferroni correction for multiple comparisons was used for comparisons of data within or between groups. Differences were considered significant when the P<.05 level was achieved. All presented values are mean±SEM.

Results

Change in ERSNA (ΔERSNA) responses to IPC administration of vehicle or 1000 µg furosemide in 36 rats with SAD and intact vagi are shown in Fig 1. IPC
administration of vehicle did not produce any changes in ERSNA (ΔERSNA=0±2%). However, IPC administration of furosemide produced sympathoexcitatory responses in 18 rats (ΔERSNA=+29±4%; *P*=.0000 versus vehicle) and sympathoinhibitory responses in 8 rats (ΔERSNA=-27±4%; *P*=.0002 versus vehicle). The average duration from time of injection to time of maximal sympathoexcitation was 8.2±0.4 seconds, and the average duration from time of injection to maximal renal sympathoinhibition was 9.7±0.9 seconds. Since both sympathoexcitatory and sympathoinhibitory responses were significantly different from the response to IPC vehicle injection, the AERSNA response to 1000 μg furosemide will be discussed as two distinct responses of either excitatory or inhibitory nature.

There were no significant differences in baseline values for mean arterial pressure (MAP), HR, and ERSNA among all groups in the present study (Table). Figs 2 and 3 show the dose-response relation between IPC dose furosemide and ΔMAP and ΔERSNA, respectively, in 10 rats with SAD and intact vagi. IPC furosemide produced significant dose-related changes in MAP and ERSNA. Furosemide 10 μg reduced MAP by 8.2±2.7 mm Hg (*P*=.06 versus vehicle) associated with a decrease in ERSNA of 14±3% (*P*=.009 versus vehicle). IPC administration of 1000 μg furosemide produced sympathoexcitatory responses in 8 rats and sympathoinhibitory responses in 2 rats. Whereas renal sympathoexcitation in response to 1000 μg furosemide IPC was seen in the absence of changes in MAP, both rats with renal sympathoinhibitory responses showed marked reductions in MAP. IPC furosemide produced no significant dose-dependent changes in HR.

The effect of bilateral vagotomy on responses to IPC injection of 2-methyl-5HT or furosemide are shown in Figs 4 and 5. Vagotomy itself did not affect MAP or HR but caused a 30±9% increase in ERSNA. Eight rats were injected IPC with 1000 μg furosemide, of which 2 showed a sympathoinhibitory response and 6 showed a sympathoexcitatory response. Whereas bilateral vagotomy inhibited both the hypotensive and the sympathoinhibitory response to 2-methyl-5HT, vagotomy did not affect either inhibitory or excitatory responses to furosemide. 2-Methyl-5HT reduced HR by 15±8 beats per minute (bpm), which was inhibited by bilateral vagotomy (ΔHR=0±0 bpm; *P*=.19).

Figs 6 and 7 show the effect of IPC lidocaine blockade on responses elicited by IPC administration of 2-methyl-5HT or furosemide. Lidocaine itself reduced baseline MAP by 29±3 mm Hg and HR by 90±9 bpm, whereas ERSNA remained unchanged. Eight rats were injected

### Mean Baseline Values

<table>
<thead>
<tr>
<th>Protocol</th>
<th>Intrapericardial Stimulation With Drug</th>
<th>Baroreflex Function</th>
<th>n</th>
<th>MAP, mm Hg</th>
<th>HR, bpm</th>
<th>ERSNA, μV·s/s</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dose response</td>
<td>Furosemide</td>
<td>SAD</td>
<td>10</td>
<td>126±1</td>
<td>360±12</td>
<td>758±102</td>
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<tr>
<td>Vagotomy</td>
<td>2-Methyl-5HT</td>
<td>SAD</td>
<td>6</td>
<td>125±4</td>
<td>356±9</td>
<td>929±141</td>
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<td>Furosemide</td>
<td>SAD</td>
<td>8</td>
<td>114±3</td>
<td>345±13</td>
<td>731±121</td>
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<tr>
<td>Lidocaine</td>
<td>2-Methyl-5HT</td>
<td>SAD</td>
<td>5</td>
<td>118±15</td>
<td>362±10</td>
<td>1254±258</td>
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<tr>
<td></td>
<td>Furosemide</td>
<td>SAD</td>
<td>8</td>
<td>118±5</td>
<td>367±17</td>
<td>636±177</td>
</tr>
<tr>
<td>Time control</td>
<td>Furosemide</td>
<td>SAD+Vagotomy</td>
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<td>117±5</td>
<td>343±14</td>
<td>494±53</td>
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<tr>
<td>Indomethacin</td>
<td>Furosemide</td>
<td>SAD+Vagotomy</td>
<td>13</td>
<td>122±4</td>
<td>349±10</td>
<td>947±133</td>
</tr>
</tbody>
</table>

n indicates number of rats; MAP, mean arterial pressure; HR, heart rate; bpm, beats per minute; ERSNA, efferent renal sympathetic nerve activity; and SAD, sinoaortic denervation. Values are mean±SEM.
Pericardial Stimulation With Furosemide

IPC with furosemide, of which 4 showed a sympathoinhibitory response and 4 showed a sympathoexcitatory response. IPC lidocaine blockade blocked both ΔMAP and ΔERSNA responses in all groups. Whereas IPC furosemide did not affect HR, 2-methyl-5HT reduced HR by 19±4 bpm, which was blocked by IPC administration of lidocaine (ΔHR=2±2 bpm; P=.01).

The effects of 1000 μg furosemide IPC before and after IPC isotonic saline administration in rats with SAD and bilateral vagotomy are shown in Figs 8 and 9. Isotonic saline induced no changes in either MAP, HR, or ERSNA. The ΔMAP and ΔERSNA responses to IPC furosemide administration were similar after IPC isotonic saline administration in rats showing either sympathoinhibitory or sympathoexcitatory responses. IPC furosemide administration did not produce any changes in HR in any group.

Figs 10 and 11 illustrate the effects of 1000 μg furosemide IPC before and after IPC indomethacin administration in rats with SAD and bilateral vagotomy. IPC indomethacin itself had no effects on MAP, HR, or ERSNA. In rats with sympathoinhibitory responses to IPC furosemide, indomethacin inhibited the hypotensive and sympathoinhibitory responses. However, in rats with renal sympathoexcitatory responses to IPC furosemide, ΔMAP and ΔERSNA responses were not affected by indomethacin. IPC furosemide caused no changes in HR in any group.

Discussion

This study demonstrates that IPC administration of 1000 μg furosemide elicits rapid and transient changes in ERSNA of either inhibitory (39%) or excitatory (33%) nature. The responses observed in SAD rats were not affected by vagotomy but were blocked by IPC lidocaine, suggesting that furosemide elicits reflex responses via stimulation of cardiac sympathetic afferents. Effective bilateral vagotomy was confirmed by the abolished hypotensive and sympathoinhibitory responses to IPC administration of
2-methyl-5HT. The type of reflex response was consistent within each animal. In rats that showed renal sympathoinhibition in response to IPC furosemide, both the hypotensive and renal sympathoinhibitory responses were inhibited by IPC indomethacin, whereas IPC indomethacin was without effect on responses in rats that showed a sympathoexcitatory response to IPC furosemide. This suggests that furosemide stimulates cardiac sympathetic afferents that result in either renal sympathoinhibition or sympathoexcitation. Cyclooxygenase metabolites mediate the reflex sympathoinhibitory responses, whereas the mechanism responsible for the sympathoexcitatory response is unknown.

Furosemide is a potent stimulator of renal prostaglandin synthesis, and several investigators have demonstrated that furosemide-induced renal synthesis of prostaglandins modulates actions of furosemide on both renal hemodynamics and tubular function. Whether furosemide stimulates prostaglandin synthesis in the heart is unknown, but the effect of IPC indomethacin on IPC furosemide-induced sympathoinhibition is compatible with furosemide-induced synthesis of cyclooxygenase-dependent metabolites in the heart. The dose of indomethacin used in this study, 10 mg/mL, is below the dose range in which indomethacin exerts actions on enzymes other than cyclooxygenase. In additional experiments, IPC injections with 1 and 10 µg of prostaglandin PGE₂ were performed in rats with SAD and bilateral vagotomy. PGE₂ 1 µg IP did not affect any parameter (n=4), whereas PGE₂ 10 µg (n=3) induced a delayed hypotensive response (−21±2 mm Hg) 2 to 3 minutes after IPC injection in the absence of any effect on ERSNA. The delayed hypotensive response to high-dose PGE₂ is likely to be due to systemic effects of this potent vasodilator prostaglandin. This suggests that PGE₂ does not exert direct effects on cardiac sympathetic afferents in the rat but that prostaglandins may facilitate the responses to other stimulatory agents. This is consistent with findings in the cat, in which PGE₂ alone exerts only marginal effects on cardiac sympathetic afferents but potentiates the stimulatory effects of bradykinin. In further support for a facilitory role of prostaglandins on cardiac sensory receptors,
acetylsalicylic acid inhibits bradykinin-induced stimulation of cardiac sympathetic afferents in dogs. A similar effect of prostaglandin synthesis inhibitors on bradykinin-induced activation of spinal afferents has been observed in afferent splanchnic nerves and afferent renal nerves. Furthermore, it has been proposed that bradykinin acts on cardiac afferents via stimulation of prostaglandin release. Thus, cardiac prostaglandins may be involved in modulating the effect of indomethacin on reflex sympathoinhibition and hypotension via cardiac sympathetic afferents after IPC furosemide administration.

In dogs, arachidonic acid, PGE₂, and PGI₂ have been shown to stimulate ventricular receptors with vagal afferents and produce a Bezold-Jarish reflex response similar to what was observed with IPC 2-methyl-5HT in this study. However, since vagotomy did not affect reflex responses to IPC furosemide, this suggests that prostaglandins may not have the same effect on vagal afferents in the rat.

IPC administration of 10 μg furosemide elicited a consistent hypertensive and sympathoinhibitory response, whereas a nonuniform response was observed after IPC administration of 1000 μg furosemide. Thus, in the low-dose range, IPC furosemide produces a uniform sympathoinhibitory and hypertensive response, which may contribute to the beneficial therapeutic effects of furosemide in congestive heart failure and hypertension. Similar to our findings with high-dose IPC furosemide, IPC bradykinin has been reported to elicit excitatory, inhibitory, and biphasic responses in the dog. In dogs and monkeys, studies on the nonuniform reflex responses to electrostimulation of cardiac sympathetic afferents have shown that pressor responses are elicited when C-type fibers are stimulated, and depressor responses are elicited when Aδ-type fibers are stimulated. Whereas the predominant response to IPC bradykinin in dog, monkey, and rabbit is a depressor and sympathoinhibitory response, IPC bradykinin elicits pressor and sympathoexcitatory responses in cats. Thus, chemical activation of cardiac sensory receptors with bradykinin elicits nonuniform reflex responses in several species as observed in the present study with IPC furosemide administration in rats.

Whether furosemide stimulates cardiac bradykinin synthesis is unknown. In the kidney, however, the diuretic response to furosemide is bluntly by a bradykinin type-2 receptor antagonist, suggesting a significant renal interaction between furosemide and bradykinin.

In conclusion, we have demonstrated that IPC furosemide elicits a reflex response mediated via cardiac sympathetic afferents that causes either renal sympathoexcitation or sympathoinhibition. The sympathoinhibitory response was associated with a decrease in MAP, and both responses were inhibited by indomethacin. Whereas prostaglandins may be involved in the sympathoinhibitory response, the mechanism responsible for the sympathoexcitatory response is unknown.

Acknowledgments

This study was supported by grants DK-15843, HL-14388, and HL-44546 from the National Institutes of Health and grants from the Veterans Administration. Dr. Petersen was supported by a grant from the Danish Heart Association, a fellowship from the Danish Medical Research Council, and an International Research Fellowship from the John E. Fogarty Center, National Institutes of Health.

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Furosemide elicits nonuniform reflex responses via cardiac sympathetic afferents.
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Hypertension. 1994;23:924-930
doi: 10.1161/01.HYP.23.6.924

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

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