Evidence for a Vasopressin System in the Rat Heart

Harald Hupf, Daniela Grimm, Günter A.J. Riegger, Heribert Schunkert

Abstract—Traditionally, a hypothalamo-neurohypophysial system is thought to be the exclusive source of arginine vasopressin (AVP), a potent antidiuretic, vasoconstricting, and growth-stimulating neuropeptide. We have identified de novo synthesis of AVP in the heart as well as release of the hormone into the cardiac effluents. Specifically, molecular cloning of sequence tags amplified from isolated, buffer-perfused, and pressure-overloaded rat hearts allowed the detection of cardiac AVP mRNA. Subsequent experiments revealed a prominent induction of AVP mRNA (peak at 120 minutes, 59-fold, \( P < 0.01 \) versus baseline) and peptide (peak at 120 minutes, 11-fold, \( P < 0.01 \) versus baseline) in these isolated hearts. Newly induced vasopressin peptide was localized most prominently to endothelial cells and vascular smooth muscle cells of arterioles and perivascular tissue using immunohistochemistry. In addition to pressure overload, nitric oxide (NO) participated in these alterations, because inhibition of NO synthase by Nω-nitro-L-arginine methyl ester markedly depressed cardiac AVP mRNA and peptide induction. Immediate cardiac effects related to cardiac AVP induction in isolated, perfused, pressure-overloaded hearts appeared to be coronary vasoconstriction and impaired relaxation. These functional changes were observed in parallel with AVP induction and largely prevented by addition of a V1 receptor blocker (10\(^{-8}\) mol/L [deamino-Pen\(^1\), O-Me-Tyr\(^2\), Arg\(^8\)]-vasopressin) to the perfusion buffer. Even more interesting, pressure-overloaded, isolated hearts released the peptide into the coronary effluents, offering the potential for systemic actions of AVP from cardiac origin. We conclude that the heart, stressed by acute pressure overload or NO, expresses vasopressin in concentrations sufficient to cause local and potentially systemic effects. (Circ Res 1999;84:365-370.)

Key Words: gene expression ■ pressure overload ■ heart ■ nitric oxide ■ vasoconstriction

An increased load of the heart such as is found in patients with aortic stenosis or arterial hypertension results in functional, structural, and molecular adaptations of both cardiac muscle and coronary vasculature. Initially, these responses to cardiac overload help to maintain cardiac output. However, prolonged activation or exhaustion of the adaptive mechanisms may result in cardiac failure.1 The activation of neurohormones, eg, angiotensin II or vasopressin, is considered to play a major role in these molecular adaptations of pressure-overloaded hearts. Interestingly, the renin-angiotensin and the vasopressin systems were traditionally thought to act exclusively in an endocrine fashion, ie, sites of generation and target tissues of functionally active peptides required the circulation for communication. Subsequently, all components of the renin-angiotensin system were localized to the heart and found to be stimulated locally by an increased load.2-4 These findings not only facilitated the implementation of pharmacological strategies but also point to more archaic functions of these neurohormonal systems, ie, local modulation of cellular growth and stress responses.1 To learn more about the rapid molecular and neurohormonal responses to cardiac overload, we compared reversely transcribed sequence tags from normal hearts and hearts exposed to elevated wall stress. We thereby uncovered a paracrine cardiac vasopressin system.

Materials and Methods

Stimulation With Wall Stress
Hearts were isolated from male Wistar rats, 9 weeks of age (Charles River, Sulzfeld, Germany), and buffer-perfused in a whole organ chamber (Langendorff-apparatus).5,6 Generation of elevated wall stress was achieved by insertion of a fluid-filled latex balloon into the left ventricle that was expanded such that the hearts were beating in a pseudo-end-diastolic state (10 mm Hg diastolic and 100 to 140 mm Hg systolic pressure, equivalent to 300-320\(\times10^3\) dyn/cm\(^2\)).5,6 The perfusion buffer consisted of a modified Krebs-Henseleit solution and was oxygenated (5% CO\(_2\)/95% O\(_2\), 35°C, pH 7.35 to 7.44), as previously described.5,6 Initially, coronary flow was adjusted to achieve a coronary perfusion pressure of 80 mm Hg. Thereafter, flow was kept constant throughout the experimental protocol. Left ventricular pressure, left ventricular end-diastolic pressure, and coronary perfusion pressure were measured by Statham dBJ transducers (Statham Instruments). Groups of control hearts (no wall stress, \( n = 10 \)) as well as groups of hearts subjected to an acute elevated wall stress (\( n = 10 \)) were perfused for 10 and 120 minutes (for differential expression of mRNA sequence tags) as well as 60 and 120 minutes (for subsequent molecular, biochemical, and functional studies).
Perfusion in the Presence of a V1 Receptor Blocker
To study functional implications of the cardiac vasopressin system, groups of 10 hearts (no wall stress and 120 minutes of wall stress) were perfused with Krebs-Henseleit buffer containing a V1 receptor antagonist (10−8 mol/L [deaminophenylalanine (DOPA), O-Me-Tyr, Arg9]-vasopressin). Dose-finding experiments revealed that this concentration was sufficient to block the coronary effects of 10−6 mol/L arginine vasopressin (data not shown).

Perfusion in the Presence of the NO Synthase Inhibitor L-NAME
To study the influence of nitric oxide (NO) on the cardiac vasopressin system, 100 μmol/L Nω-nitro-L-arginine methyl ester (L-NAME) was added to the perfusion buffer. Groups of 10 hearts (control, 60 minutes and 120 minutes of wall stress) were perfused with and without L-NAME for comparison both with and without wall stress.

Differential Display
Reversely transcribed mRNA (cDNA) was systematically amplified by reverse transcriptase amplification (RTA) from normal and hypertrophic hearts exposed to elevated wall stress using a differential display assay. Each reverse transcription (RT) reaction was performed with a primer anchored at the 5′-end of poly(A) tails plus an arbitrary upstream primer. RT was performed on each RNA sample using 500 ng total RNA in 1× RT buffer, 10 mmol/L DTT, 20 μmol/L of each deoxynucleotide triphosphate, 1 μmol/L T12N(C,G,T) anchored primer, and 200 U Moloney murine leukemia virus RT (Life Technologies) per 20 μL of reaction volume. Amplification of the cDNA was performed under the following conditions: 94°C, 1 minute followed by 40 cycles of 94°C, 30 seconds; 54°C, 2 minutes; 72°C, 30 seconds, and ending with 72°C, 5 minutes (1 μL RT reaction mix, 1× PCR buffer, 1 μmol/L T12N(C,G,T) anchored primer, 1 μmol/L arbitrary primer, 8 μmol/L dNTPs, 0.25 μCi [35S]-dATP, and 1.25 μL Taq DNA polymerase [Boehringer-Mannheim] per 10 μL reaction volume). The assays were realized with 60 different primer combinations. The products were radioactively labeled with [35S]-dATPωS (Amersham) during PCR and fractionated by denaturing polyacrylamide gel electrophoresis (8% polyacrylamide/6 mol/L urea gel). Differentially expressed bands were eluted (Crush & Soak buffer containing 0.5 mol/L NH4OAc, 10 mmol/L MgOAc, 1 mmol/L EDTA, and 1% SDS, cloned (Advantage PCR cloning kit, Clontech), and sequenced (Sequrise). The sequences were compared with GENEMBLE (GCG Genetics Computer Group).

RNA Analysis
Randomly reverse-transcribed mRNA (cDNA) was amplified by PCR in the presence of specific primers GCTACTCTCGACGCACCG (91–108) and GCTACTCTCGAGCCACCG (350–367) (exon 1 and 3, product size = 275 bp). The PCR products were carried out using 3 μL RT reaction mix, 1× PCR buffer, 0.5 μmol/L sense primer, 0.5 μmol/L antisense primer, 2 μmol/L dNTPs, and 1.25 U Taq DNA polymerase per 20 μL of reaction volume. PCR conditions were as follows: 94°C, 30 seconds; 54°C, 1 minute; 72°C, 30 seconds for 32 cycles and an additional 10 minutes at 72°C. The final reaction products were electrophoresed on 3% agarose gels, stained with ethidium bromide, visualized under UV light, and quantified by densitometry. The amplification products of cardiac vasopressin were compared with those of an externally added non-homologous standard of vasopressin (Sigma) were reconstituted to 50 μL, and 50 μL of vasopressin antibody (final dilution of 1:15 000) was added. Thereafter, 5500 cpm of 125IArg8-Vasopressin (specific activity 2200 Ci/mmol, NEN Life Science Products) was diluted in 50 μL assay buffer and added, followed by a 40-hour incubation at 4°C. Separation of bound ligand was then performed on ice by adding 250 μL undiluted sheep serum (Biozol Diagnostica) and 250 μL 16% PEG-8000 (Sigma) diluted in assay buffer, followed by centrifugation. The pellets were counted in a gamma counter (Cobra II, Canberra Packard).

Immunohistochemistry
Frozen tissues were sectioned at 5 μm and fixed with acetone (−20°C) for 10 minutes. The indirect peroxidase technique was used to visualize antigen-antibody complexes. Incubation with the first antibody (vasopressin, ICN) was followed by incubation with the second antibody (swine-anti-rabbit, Daco Patts). After repeated washing with PBS, the sections were dehydrated and embedded with Entellan (Merck). All sections were visualized by light microscopy using an oil immersion objective with a calibrated magnification of ×400.

Statistical Analysis
All data are shown as mean±SEM. Statistical analysis between groups was performed by unpaired t test or ANOVA analysis for comparison of 3 or more groups. A value of P<0.05 was considered significant.

Results
Differential Display Assay
Of 14 000 expressed sequence tags, 54 were found only in hearts exposed to elevated wall stress. Sequencing of these bands revealed that the vast majority (50) represented currently unknown genes. However, the primer combination T12N(C,G,T) anchored primer and GAGTTCGACAG (arbitrary primer) allowed detection of a prominent new band that was found to be 95% homologous to a 200-bp stretch of rat vasopressin.

Increased Vasopressin mRNA Levels
To follow up on this observation, the upregulation of the cardiac vasopressin was verified by specific semiquantitative RT-PCR. After 60 and 120 minutes of isolated perfusion and high wall stress, vasopressin mRNA levels increased to 5.8 and 14.3 pg/μg total RNA. Compared with the low expression levels found in normal hearts, this related to a 24- and 59-fold induction of vasopressin mRNA after 60 and 120 minutes of left ventricular pressure overload, respectively (P<0.005, Figure 1A and 1B).

Increased Vasopressin Peptide Levels
Using a sensitive radioimmunoassay, we discovered that 60 and 120 minutes of elevated wall stress related to a 6- and 11-fold induction of cardiac vasopressin at the peptide level, respectively (P<0.05, Figure 1C). For comparison, we used hearts that had been buffer-perfused for 15 minutes and thus were essentially free of plasma contamination. Furthermore, immunohistochemistry revealed a prominent vasopressin induction after 120 minutes of stimulation in the vascular wall of arterioles and the perivascular interstitium (Figure 2).
cell types with the strongest signals for vasopressin immunostaining included endothelial cells and vascular smooth muscle cells of arterial vessels (100 to 400 μm²). Likewise, endothelial cells of capillaries displayed vasopressin immunostaining. Moreover, increasing vasopressin concentrations were detected in the coronary effluents after 60 and 120 minutes of perfusion with elevated wall stress (Figure 3).

Effects of NO
Because some induction of vasopressin occurred even in the absence of elevated wall stress in these isolated hearts, we also explored other mechanisms that might result in cardiac/coronary vasopressin induction. Particularly, we perfused hearts in the presence of an inhibitor of the NO synthase (L-NAME) with and without concomitant pressure overload. This approach was selected because NO can be liberated in isolated perfused hearts and is potentially involved in central vasopressin regulation.12 As can be seen in Figures 1 and 3, the induction of vasopressin mRNA and peptide levels, as well as their release, was largely prevented when hearts were perfused with wall stress for 120 minutes in the presence of L-NAME.

Functional Studies
The potential hemodynamic effects of cardiac vasopressin synthesis were also studied in these isolated perfused rat hearts. Specifically, coronary perfusion pressure (at constant coronary flow) and left ventricular systolic and end-diastolic pressures (at constant left ventricular balloon volume) were recorded in 15-minute intervals. As can be seen in Figure 4 and the Table, a progressive increase in coronary perfusion pressure and left ventricular end-diastolic pressure was observed after 60 minutes of perfusion with elevated wall stress, ie, when cardiac vasopressin peptide levels were elevated in these isolated hearts. When these experiments were conducted in the presence of a vasopressin receptor V₁ inhibitor ([deamino-Pen₁, O-Me-Tyr², Arg⁸]-vasopressin, 10⁻⁸ mol/L, Sigma), the spontaneous increase in coronary perfusion pressure and the spontaneous increase in left ventricular end-diastolic pressure normally observed during long-term perfusion of rat hearts in the Langendorff apparatus were largely prevented (Figure 4 and the Table).

Discussion
The present study demonstrates, for the first time, the existence of a cardiac vasopressin system in rat hearts.

Figure 1. A, Vasopressin mRNA of untreated hearts (control) and mRNA of hearts stimulated with wall stress for 60 and 120 minutes. Vasopressin mRNA levels of hearts exposed to no wall stress or stimulation with and without wall stress for 120 minutes in the presence or absence of 100 μmol/L L-NAME are shown. To quantify the vasopressin mRNA elevation, different concentrations of the truncated vasopressin plasmid are shown.

Hupf et al February 19, 1999 367

There is a significant increase of the vasopressin mRNA expression after 60 and 120 minutes of wall stress. Perfusion of 120 minutes without wall stress resulted in significantly lower vasopressin levels. Likewise, L-NAME could partially inhibit the increase in vasopressin mRNA. B, Quantitative analysis of 10 hearts in each group. Comparison by ANOVA revealed a significant upregulation of vasopressin mRNA after stimulation with wall stress (24- and 59-fold upregulation after 60 and 120 minutes, respectively). No wall stress (flaccid balloon) or the addition of NO synthase inhibitor (L-NAME) partially blocked vasopressin synthesis. Complete blockade was achieved by perfusion with a flaccid balloon, in combination with L-NAME (10⁻⁸ mol/L). C, Cardiac vasopressin peptide levels in the same groups as displayed in panel B.
exposed to pressure overload. Specifically, using isolated perfused rat hearts and differential display technology, we demonstrated the presence of vasopressin mRNA in cardiac tissue. This observation, coupled with the identification of vasopressin immunoreactivity and elevated peptide concentrations after exposure to elevated wall stress, strongly suggests that under certain conditions, the heart may participate in the generation of vasopressin.

This observation is interesting because patients with cardiac dysfunction are known to present with elevated vasopressin levels. In fact, the elevation of vasopressin in these patients has important prognostic implications. Subsequently, it was hypothesized that activation of vasopressin—like the activation of other neurohormonal systems—is interpolated in the vicious circle that is started by cardiac overload and ultimately ends in threatening heart failure. Elevated plasma vasopressin levels have also been observed in experimental animals with left ventricular hypertrophy due to aortic stenosis. The cellular sources of increased vasopressin levels in patients with congestive heart failure or rats with severe left ventricular hypertrophy remain unclear.

Thus far, the brain, where vasopressin can act as a local neurotransmitter, is considered to be the principal locus of vasopressin synthesis. Little vasopressin generation has been reported in endocrine tissues, including ovary, testis, and endothelial cells of pulmonary, renal, and mesenteric arteries.
By contrast to restricted vasopressin generation sites, vasopressin receptors and actions are widely distributed and diverse, mainly mediating vasoconstricting, antidiuretic, and growth-promoting effects. Although blood pressure and osmoregulation are considered to be the main functions of the neurohypophysial vasopressin system, the peptide may also participate in the regulation of cardiac function, perfusion, and cardiac neurohormone secretion.

The most prominent vasopressin effects on cardiac physiology are coronary vasoconstriction and impaired relaxation. In the context of the present experiments, these effects are interesting because a number of investigators, including our group, had observed that vasoconstriction and impaired relaxation occur “spontaneously” in isolated working hearts undergoing prolonged perfusion. Thus far, this phenomenon was largely attributed to a progressive tissue edema resulting from the use of hypoprotective perfusion buffers. The present data offer an additional explanation, namely, the local induction of vasopressin. In fact, progressive increases in coronary perfusion pressure and left ventricular end-diastolic pressure were observed after 60 minutes of perfusion with elevated wall stress, ie, in parallel with the cardiac vasopressin peptide induction. Even more significant, when these experiments were conducted in the presence of a vasopressin inhibitor, the increase in coronary perfusion pressure and the deterioration of diastolic parameters could be partially prevented. Thus, we have reason to believe that the local induction of vasopressin in the heart is functionally relevant and that it explains some of the functional changes observed during prolonged ex vivo perfusion. Although not explored in the present investigation, local vasopressin production may also translate to growth induction of cardiac myocytes, as well as endothelin and atrial natriuretic peptide release, ie, responses that have been observed in pressure-overloaded as well as vasopressin-stimulated hearts.

Guided by the finding that some vasopressin induction occurred even in the absence of elevated wall stress, we also explored other potential mechanisms involved in vasopressin stimulation. We turned our attention to factors that may rapidly modulate gene expression in the vascular wall, ie, the site where vasopressin immunoreactivity was found. In this context, 2 recent observations led us to hypothesize that NO generation is involved in the cardiac induction of vasopressin. First, NO had been reported to be induced in isolated working hearts. Second, NO is known to interact with vasopressin synthesis. Specifically, vasopressin is known to be synthesized in high concentrations when NO levels are elevated, an effect that can be sharply corrected by the use of the NO synthase inhibitor L-NAME. Indeed, an NO synthase inhibitor (L-NAME) largely prevented the induction of vasopressin in the pressure-overloaded hearts. Thus, in addition to the local induction of vasopressin, the present data provide indirect evidence for the functional relevance of NO synthesis in isolated pressure-overloaded hearts. In a broader context, it appears that the rapidly acting vasodilator NO induces a slow counterbalancing process, namely vasopressin induction. This finding may be of utmost relevance for the pharmacodynamics of drugs that stimulate the local release of NO, ie, nitroglycerin. These drugs have in common a progressive loss of their vasodilatory action within 12 to 36 hours. Moreover, patients undergoing prolonged treatment with nitroglycerin were found with increased vasopressin plasma concentrations. Thus far, this observation was explained as a compensatory reaction to the blood pressure decrease seen with the use of these drugs. Certainly, the present finding of an NO-mediated vasopressin induction in the coronary vasculature offers another explanation of nitrate tolerance or, ie, the vascular induction of vasoconstricting factors in response to chronic NO donation.

Finally, the present data allow the speculation that cardiac vasopressin, synthesized and released on stimulation with cardiac pressure overload or NO, displays systemic effects because increasing concentrations of the neurohormone were detected in the coronary effluents of these isolated perfused hearts. The spillover of vasopressin was quite sizable, resulting in a vasopressin release into the coronary effluents that approached concentrations found in normal plasma. Given the half-life of the peptide (8 minutes), these quantities may be sufficient to result in fluid retention or other systemic vasopressin actions. It is noteworthy, in this regard, that the renal medullary V2 receptors are highly sensitive to vasopressin and respond at low concentrations (Bmax 10−9 mmol/mg of protein). Additional investigations must explore the potential of systemic effects of coronary-derived vasopressin in intact animals. Specifically, it will be of importance to investigate cardiac vasopressin generation in disease models of heart failure or pressure overload and ultimately to explore its implications in patients with these conditions.

Taken together, exploration of differentially displayed vasopressin mRNA of isolated, perfused, pressure-overloaded rat hearts allowed the detection of multiple newly induced sequence tags, suggesting a profound reorganization of cardiac gene expression. Most significantly in this situation, cardiac vasopressin mRNA and peptide induction were uncovered using this approach. Local implications of such a local vasopressin system may include coronary vasoconstriction, impaired relaxation, or as previously shown, growth induction of cardiac myocytes. Indirect vasopressin effects may add to the scenario because vasopressin has been shown to modulate the release of cardiac atrial natriuretic peptide and endothelin-1. Finally, the present data allow the speculation that cardiac vasopressin, synthesized and released in sizable quantities on stimulation with cardiac pressure overload and NO, displays systemic effects.

<table>
<thead>
<tr>
<th>Stimulation</th>
<th>Time, min</th>
<th>LVP, mm Hg</th>
<th>LVEDP, mm Hg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wall stress plus vehicle</td>
<td>0</td>
<td>130.3±6.9</td>
<td>10±1</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>155.5±6.4*</td>
<td>11.7±2.2 n.s.</td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>142.3±6.4*</td>
<td>23.3±5.0*</td>
</tr>
<tr>
<td>Wall stress plus V1 blocker</td>
<td>0</td>
<td>136.7±7.3</td>
<td>10±1</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>142.0±7.3 n.s.</td>
<td>9.2±1.2 n.s.</td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>139.4±6.5 n.s.</td>
<td>14.2±2.2 n.s.</td>
</tr>
</tbody>
</table>

*P<0.05 vs baseline (0 min). LVP indicates left ventricular pressure; LVEDP, left ventricular end-diastolic pressure; and n.s., not significant.
Acknowledgments
This study was supported by the Deutsche Forschungsgemeinschaft (DFG Schu 672/3-1, 9-1, 10-1, and 12-1) and the Bundesministerium für Forschung und Technologie (to H.S.). We thank Susanne Kürzinger and Ingrid Kirst for excellent technical assistance.

References
Evidence for a Vasopressin System in the Rat Heart
Harald Hupf, Daniela Grimm, Günter A. J. Riegger and Heribert Schunkert

Circ Res. 1999;84:365-370
doi: 10.1161/01.RES.84.3.365

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1999 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/84/3/365

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org//subscriptions/