Enhanced Cardiac Contractility After Gene Transfer of V2 Vasopressin Receptors In Vivo by Ultrasound-Guided Injection or Transcoronary Delivery

Hans-Jörg Weig, MD; Karl-Ludwig Laugwitz, MD; Alessandra Moretti, PhD; Kai Kronsbein, BS; Christian Städele, MD; Stefan Brüning, MD; Melchior Seyfarth, MD; Thomas Brill, MD; Albert Schömig, MD; Martin Ungerer, MD

Background—Systemic levels of arginine vasopressin (AVP) are increased in congestive heart failure, resulting in vasoconstriction and reduced cardiac contractility via V_1 vasopressin receptors. V_2 vasopressin receptors (V2Rs), which promote activation of adenylyl cyclase, are physiologically expressed only in the kidney and are absent in the myocardium. Heterologous expression of V2Rs in the myocardium could result in a positive inotropic effect by using the endogenous high concentrations of AVP in heart failure.

Methods and Results—We tested gene transfer with a recombinant adenovirus for the human V2R (Ad-V2R) to stimulate contractility of rat or rabbit myocardium in vivo. Ultrasound-guided direct injection or transcoronary delivery of adenovirus in vivo resulted in recombinant receptor expression in the myocardial target area, leading to a substantial increase in [3H]AVP binding. In 50% of the cardiomyocytes isolated from the directly injected area, single-cell shortening measurements detected a significant increase in contraction amplitude after exposure to AVP or the V2R-specific desmopressin (DDAVP). Echocardiography of the target myocardial area documented a marked increase in local fractional shortening after systemic administration of DDAVP in V2R-expressing animals but not in control virus–treated hearts. Simultaneous measurement of global contractility (dP/dt_{max}) confirmed a positive inotropic effect of DDAVP on left ventricular function in the Ad-V2R–injected animals.

Conclusions—Adenoviral gene transfer of the V2R into the myocardium increases cardiac contractility in vivo. Heterologous expression of cAMP-forming receptors in the myocardium could lead to novel strategies in the therapy of congestive heart failure by bypassing the desensitized β-adrenergic receptor–signaling cascade. (Circulation. 2000;101:1578-1585.)

Key Words: adenovirus ■ gene transfer ■ vasopressin ■ myocardium

Congestive heart failure is characterized by increased sympathoadrenergic drive, stimulation of the renin-angiotensin-aldosterone system, and augmented release of several peptide hormones, primarily arginine vasopressin (AVP) and atrial natriuretic peptide. The increase in AVP and catecholamines is inversely correlated with the prognosis of patients with congestive heart failure.1,2 In response to adrenergic stimulation, however, the failing myocardium cannot build up a sufficient contractile force because β-adrenergic receptors are functionally inactivated.3,4

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The current study tested the feasibility of adenoviral gene transfer in vivo to improve cardiac contractility by overexpressing a positive inotropic receptor, the recombinant V_2 vasopressin receptor (rV2R). Physiologically, only vasopressin receptors (V1ARs) are expressed in the myocardium, where they exert a weak, negative inotropic effect. In contrast, V2Rs are expressed exclusively in the kidney, where they couple to cAMP formation. Therefore, we wished to induce a genetic receptor subtype shift in the myocardium by overexpressing the rV2Rs. In our previous study in cardiomyocytes, we had shown that rV2Rs are not downregulated by agonists.5 Therefore, the heterologous receptor can be assumed to retain most of its function even at prolonged agonist exposure. rV2Rs could also be expected to exert a positive inotropic effect in the failing heart, since in this condition, the endogenous concentrations of AVP are markedly increased.

Adenoviral gene transfer into the myocardium in vivo has been accomplished for several marker genes after direct injec-
tion into the ventricular wall or after intravasal application into the coronary arteries. However, all of these approaches were hampered by a relatively low efficiency of gene transfer. No studies have described the expression of transgenes with inotropic action, so that no measurements of transgene function in vivo were carried out. One publication hypothesized increased cardiac vascularization after gene transfer of fibroblast growth factor-5, without providing data on the cell type of transgene expression or the efficacy of gene transfer. Recently, Hajjar and colleagues demonstrated dampened contractility after adenoviral gene transfer of phospholamban by open-chest direct injection into the ventricle with simultaneous pulmonary and aortic clamping.

For our study, however, we wished to test an easier and hence, more practicable approach for transgene delivery, ie, intracoronary administration after percutaneous catheterization. This concept seemed feasible, after a recent study in a rabbit heart Langendorff preparation showed a much higher efficiency for transcoronary gene transfer ex vivo after pharmacological modification of the endothelial barrier. We used a modification of this protocol to achieve transcoronary gene delivery in vivo. The aim of the study was to explore novel approaches for in vivo gene transfer of recombinant adenoviral vectors encoding the human V2R to improve cardiac inotropy in adult myocardium in vivo.

Methods

Construction and Purification of Recombinant Adenovirus

Generation of recombinant (E1-deficient) adenoviruses encoding the human V2R (Ad–V2R) or the nuclear-targeted mutant β-galactosidase (Ad–β-Gal) has already been described. In addition, a bicistronic adenovirus encoding both V2R and green-fluorescent protein (Ad–V2R-GFP) was generated after kind provision of plasmids by Dr He (Howard Hughes Medical Institute, Baltimore, Md). All transgenes were driven by cytomegalovirus promoters. The viruses were amplified in large stocks and purified through CsCl gradients. Adenoviral titers were determined by plaque titration on HEK 293 cells.

Ultrasound-Guided Direct Adenovirus Injection Into Rat Myocardium

Twelve- to 16-week-old male Wistar rats (weight 420 ± 23 g; from Charles River, Munich, Germany) were used. The project was approved by the institutional ethics review board. The rats were anesthetized with midazolam (2 mg/kg SC) and medetomidin (150 μg/kg SC) and placed in a supine position. For echocardiography, a 7.5-MHz probe was fixed on a tripod. A 31-gauge needle was placed, under ultrasound control, into the left ventricular inferior wall. The probe suspension (150 μL, 10^10 pfu) was slowly injected in a fractionated manner.

Transcoronary Delivery of Recombinant Adenovirus to Rabbit Myocardium

New Zealand White rabbits (weight 3.6 ± 0.3 kg; from Charles River) received medetomidin (100 μg/kg IM), propofol (5 mg · kg^{-1} · h^{-1} IV), and a bolus of fentanyl (10 μg/kg IV). They were intubated, ventilated, and monitored for ECG, echocardiography, and pressure throughout the experiment. Through a 5F sheath in the carotid artery, a JR-5F human diagnostic catheter (Cordis) was placed opposite the left coronary ostium. Then, a 2.4F Microferret (Cook) catheter was selectively introduced into the proximal circumflex artery. Three milliliters of Krebs–Ringer solution containing 10 μmol/L serotonin and 1.25 mmol/L calcium was infused for 2 minutes, followed by infusion of 1.5 mL of 10^{10} pfu of purified adenovirus over 2 minutes. No signs of ischemia or ischemic damage were registered either during the intervention (continuous ECG recording) or at necropsy.

Myocardial Contractility Measurement by Echocardiography and Intraventricular Tip Catheterization

Left ventricular contractility was examined before and 72 hours after adenoviral gene transfer. The rats or rabbits were anesthetized as described before. Echocardiographic M-mode recording was carried out as described in previous studies. In addition, ECG and blood pressure were monitored continuously. After preparation of the right carotid artery, a Millar 2.5F tip catheter connected to a differentiating device (Hugo Sachs) was placed in the left ventricle. After definition of basal contractility and left ventricular pressure, 200 μL of NaCl (0.9%) was injected as a negative control. After a sufficient equilibrium period, desmopressin (DDAVP) was injected in doses of 10^{-11} mol and 10^{-10} mol (rats) or 4×10^{-12} to 4×10^{-8} mol (rabbits) to achieve estimated intracoronary concentrations ranging from 10^{-9} mol/L to 10^{-3} mol/L (rats) or 10^{-10} to 10^{-6} mol/L (rabbits), respectively. Measurements were carried out 1 minute after each injection.

Preparation of Adult Ventricular Cardiomyocytes

As previously described, single, calcium-tolerant ventricular cardiomyocytes were isolated for in vitro infection or were prepared from the target area after injection of adenovirus into rat hearts in vivo. In vitro infection (at a multiplicity of infection of 80 pfu/cell) was carried out after 6 hours in culture. The contraction amplitude of cardiomyocytes was determined by using an electro-optical monitoring system as described before.

Radioligand Binding

The rat or rabbit hearts that had been examined physiologically in vivo were excised, cut into pieces, resuspended in 5 mmol/L Tris-HCl (pH 7.4) and 2 mmol/L EDTA, and homogenized. The homogenate was centrifuged at 1000g for 15 minutes, and the supernatant was centrifuged twice at 100 000g for 30 minutes.
The resulting membrane pellet was resuspended in Tris-HCl-buffer, pH 7.4. The radioligand binding experiment was carried out as described before.3

β-Gal Expression
Cardiomyocytes infected with Ad–β-Gal were fixed and stained as previously described.5 Frozen hearts were cut into slices at a thickness of 7 μm in a freeze microtome, stained similarly, and counterstained by hematoxylin-eosin.

Data Analysis
Data represent the mean±SD or, where appropriate, mean±SEM. Data were compared by ANOVA for repeated measurements with respect to equal doses in all groups, followed by a post hoc Scheffe’s test (all results except those of Figure 3) or by Student’s t test with a 2-tailed distribution (Figure 3).

Results
Single Cardiomyocytes Infected In Vitro
Figure 1 shows the effect of the physiologic agonist AVP and of the V2R-specific agonist DDAVP on externally paced, isolated ventricular cardiomyocytes infected in vitro. Neither AVP nor DDAVP changed the contraction amplitude in control cells, whereas both agents stimulated Ad-V2R-infected cells to the same extent as β-adrenergic agonists. For both agents, we observed subnanomolar EC50 values, well below the concentrations needed for stimulation of the native present V1ARs. Similarly, DDAVP increased contraction amplitude in green-fluorescent Ad–V2R-GFP–infected cells, whereas no effect was observed in nonfluorescent cells (positive and negative cells in Figure 2B).

Ultrasound-Guided Injection of Adenovirus Into the Myocardium
Ad-V2R, Ad–β-Gal, or Ad–V2R-GFP was used for somatic gene transfer into the myocardium. We established a method to insert an echogenic needle subxyphoidially, guided by echocardiography. Needle placement and the correct injection site could be monitored in real time. Misplaced injections could be distinguished by the absence of an echo contrast enhancement in the target area. In a pilot study that compared echo imaging with histological analysis, we found that echocardiography showed the correct placement of gene injection with a specificity of 100% (n=6), whereas misplaced injections were also correctly predicted (n=6). Figure 2 shows the macroscopic (Figure 2C) and microscopic (Figures 2D and 2E) images of hearts injected with Ad–β-Gal (Figures 2C and 2E) or controls (Figure 2D). In a series of reproducible experiments, approximately one fifth of the total ventricular myocardium could be reached by a single, fractionated injection, as estimated from macroscopic staining of serial slices of the myocardium (Figure 2C). Specific staining showed a small, central necrotic focus along the needle track of the injection area and a markedly larger area of viable transgene-expressing myocardium surrounding this track.

There was no evidence of β-Gal expression in the livers, spleens, aortas, or lungs of the animals that received intracardiac injections of recombinant adenovirus. Fluorescence imaging of sections of a heart injected with bicistronic Ad–V2R-GFP revealed transgene expression of GFP and thus, rV2R, throughout the myocardium (Figure 2F).

Percutaneous Transcoronary Administration of Adenovirus
Ad-V2R and Ad–β-Gal were also applied to rabbit myocardium by transcoronary infusion after prior intracoronary administration of 10 μmol/L serotonin at a low calcium concentration (1.25 mmol/L). Sections through the posterior wall of rabbit hearts 3 days after administration of Ad-V2R or Ad–β-Gal are shown in Figures 2I and 2H. By using our method, approximately one fifth of the left ventricular myocardium could be reached, as estimated from macroscopic staining of serial slices of the myocardium (Figure 2G). Transgene expression was reproducibly restricted to the lateroposterior wall, corresponding to the perfusion bed of the circumflex artery in rabbits.

V2R Expression in the Target Area of Intracardiac Gene Transfer
Similar to Ad–β-Gal, Ad-V2R and Ad–V2R-GFP were injected directly into the inferior wall of rat hearts or applied transcoronarily into the lateroposterior wall of rabbits in vivo. In all cases, radioligand binding with [3H]AVP documented expression of rV2Rs after infection with Ad-V2R. The average Bmax value in the target myocardium reached 3 times the value of control areas in the same animals or in Ad–β-Gal–infected animals (Figure 3). In the control areas, [3H]AVP binding results from native expression of V1AR.

Time Course of Transgene Expression
Adenoviral gene transfer led to maximum transgene expression 6 days after infection (estimated efficiency of 50%), with a subsequent, gradual reduction of expression for the following weeks (estimated at 25% after 2 weeks, 3% to 4% after 3 weeks, and <1% after 4 weeks). This was paralleled by an increasing infiltration of inflammatory cells (negligible 2 days after infection, pronounced after 2 and 3 weeks, and decreasing after 4 weeks).

Isolated Cardiomyocytes From the Target Myocardium After In Vivo Gene Transfer
Robust expression of the 2 adenoviral transgenes was demonstrated after myocardial infection of rat hearts in vivo and subsequent isolation of ventricular cardiomyocytes from the target myocardial area. After in vivo infection with 107 pfu Ad–β-Gal and subsequent staining with X-gal, ~50% of the cardiomyocytes reproducibly showed positive staining. To investigate the effect of rV2R expression on the function of single cardiomyocytes after in vivo transfer, we measured the

Figure 2. A and B, Light microscopy (A) and green fluorescence (B) of positive and a few negative (arrow) cardiomyocytes infected with Ad–V2R-GFP. C, Macroscopic slices of a rat heart injected with Ad–β-Gal (slice thickness, 7 μm; width between slices, 200 μm). D, E, and F, Fluorescence image of tissue slices of a rat heart injected directly with Ad–V2R-GFP (F). G, H, and I, Myocardial tissue slices of rabbit hearts that had received transcoronary administration of control virus (G) or Ad–β-Gal (H and I) and were stained with X-gal.

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contraction amplitude of rat cardiomyocytes isolated from the target and control areas and from control virus–injected hearts. All investigated cardiomyocytes showed a clear increase in contraction amplitude after administration of 10^{-7} mol/L isoproterenol. Ad–V2R-GFP–infected cardiomyocytes that displayed green fluorescence (n = 10) showed a marked increase in contraction amplitude in response to DDAVP, whereas nonfluorescent cells did not (n = 8; not shown). From these experiments, we concluded that the capacity of cardiomyocytes to be stimulated by DDAVP clearly depended on expression of rV2R in an individual cell. In 50% of the cardiomyocytes from the Ad-V2R–injected area, we detected a significant increase in contraction amplitude after application of DDAVP, starting at a concentration of 10^{-11} mol/L (Figure 4). The maximum increase in contraction amplitude was comparable to the results obtained with in vitro–infected cardiomyocytes and reached a similar level as did stimulation of native β-adrenergic receptors with isoproterenol. However, the remaining 50% of the cardiomyocytes isolated from the target area did not respond to the addition of DDAVP (“negative cells” in Figure 4), demonstrating that they had no or insufficient transgene expression to produce a functional effect on contraction.

**Effect of Ad-V2R Infection on Cardiac Function In Vivo**

To examine the effect of V2R expression in the intact myocardium in vivo, we measured local echocardiographic fractional shortening before and after the systemic application of DDAVP. Validation of the echocardiographic measurements has been extensively described.12 Neither in rats nor in rabbits did cardiac frequency and systemic blood pressure differ significantly at any concentration of DDAVP, as documented by simultaneous ECG recording (also see the Table). Figure 5A shows the mean values of local fractional shortening in the injection area for control- and Ad-V2R–injected rats. A clear increase in fractional shortening occurred in the inferior wall of Ad-V2R–injected animals, whereas no such effect was seen in control virus–injected animals. Left ventricular dP/dt_{max} as a measure of global ventricular contractility was determined by placing a tip catheter in the left ventricle. This approach aimed at detecting changes in global left ventricular contractility to complement the echocardiographic information on local myocardial contractility. After injection of DDAVP, an increase in dP/dt_{max} was observed in the V2R-expressing rats, whereas no change was seen in the control group (Figure 5B). Also after transcoronary administration of Ad-V2R in rabbits, we observed significant increases in fractional shortening, relative systolic thickening of the posterior wall (calculated according to Reference 13), and left ventricular contractility, which were all absent in rabbits that had received control virus. The results are shown in the Table.

**Discussion**

In the present study, we have demonstrated effective functional expression of rV2Rs in the myocardium after adenoviral gene transfer in vivo by either direct injection or transcoronary administration. Thus, we show that recombinant, heterologous receptors are capable of enhancing cardiac contractility in vivo. These results complement a previous study, in which we had shown that infection of cultured hearts...
cardiomyocytes with Ad-V2R in vitro led to a robust transgene expression.\(^5\)

Several previous studies have described the successful transfer of recombinant adenoviruses into the myocardium either by direct injection or by intracoronary infusion.\(^6,7\) However, all of these approaches were hampered by a relatively low efficiency of gene transfer. None described the expression of transgenes with inotropic action, so that no measurements of transgene function in vivo were carried out. For the first time, we have shown in vivo somatic gene transfer with sufficient efficacy to achieve a positive inotropic effect of the transgene after either direct intracardiac injection or transcoronary delivery.

For this purpose, we improved both methods. Direct virus injection with high specificity was made possible by using a fractionated injection under control of a highly resolving ultrasound probe. This technique enabled us to control both the injection site and the success of the injection and to exclude misplaced injections by the absence of echo contrast enhancement in the target myocardial area. Transcoronary gene delivery was improved by using the protocol proposed by Donahue and collaborators\(^10\) in a recent seminal ex vivo study that showed a 96% transduction efficacy by infusing serotonin in a calcium-depleted solution before virus administration to modify the endothelial barrier.

The success of infection was tested by histological examination and X-gal staining for β-Gal expression and by radioligand binding for V2R expression. Membranes from noninfected myocardium showed endogenous ligand binding for [3 H]AVP due to the cardiac expression of native V1ARs. In contrast, [3 H]AVP binding was increased 3-fold in cardiac membranes prepared from the target area by adenoviral infection with Ad-V2R, we were thus able to demonstrate Widespread myocardial expression of rV2Rs in situ was documented by using a bicistronic Ad–V2R–GFP. After adenoviral infection with Ad-V2R, we were thus able to induce a genetic receptor subtype shift by overexpressing...
rV2R versus native V1R in the target myocardial area by a factor of 2 to 3:1.

In addition, we have demonstrated a functional in vivo effect of the recombinant receptor proteins after injection of selective V2 agonists. The V2-selective compound DDAVP had to be used for these in vivo experiments because the physiological agonist, AVP, induces strong, coronary vasoconstriction and hence, negative inotropy, when given intracoronarily.14 In addition, DDAVP did not show any chronotropic effects. Moreover, DDAVP is virtually devoid of peripheral vascular effects, and its effects on renal fluid retention only occur 10 to 20 minutes after systemic administration.

Echocardiographically, we determined a clear increase in regional fractional shortening of the target myocardial area after the administration of the receptor agonist. This increase must have been caused specifically by rV2Rs, because it was absent in control animals or in control virus–injected animals. On measuring dP/dt_{max} in the left ventricle simultaneously, we also detected a significant increase in global contractility, which was also absent in control animals or in control virus–injected animals.

By studying individual cardiomyocytes isolated from the target injection area of rat hearts, we were able to demonstrate that the observed increase in contractility was mediated by the cardiomyocytes and to exclude artificial effects mediated by other cell types, such as fibrocytes. After exposure to AVP, DDAVP, or both, Ad-V2R–infected ventricular cardiomyocytes showed a dose-dependent increase in their contraction amplitude. In the in vivo–infected myocardial target area, this was true for 50% of the isolated cardiomyocytes, thus proving high efficacy of gene transfer. Because of the better transfection conditions that can be reached in vitro than in vivo, more V2 receptors/myocytes will be expressed after in vitro infection. This fact should explain the difference in maximum average fractional shortening between the in vivo and in vitro groups, since a higher receptor/myocyte density will lead to a more pronounced effect on contractility.

Obviously, rV2Rs were coupled to the endogenous G_{s}/adenyl cyclase system with sufficient stringency to affect cardiac function. The effect started at a concentration of 0.5 nmol/L, which is correlated to the physiological AVP concentrations in vivo in the circulation of patients with congestive heart failure.2 In addition, wall stress–triggered local myocardial synthesis of vasopressin has been described recently.15 Overexpressing V2Rs in the myocardium of patients with heart failure might therefore turn the negative inotropic effect of high levels of AVP into a beneficial, positive inotropic effect. This article represents the first report about the functional modulation of the cardiac contractile apparatus in vivo by overexpression of a heterologous, positive inotropic receptor. It might therefore be possible to use this strategy to modulate different functions of target organs in a wide variety of pathological states.

In heart failure, several alterations of the β-adrenergic pathway occur, including downregulation of β1-adrenergic receptors3 and uncoupling of the remaining β-adrenergic receptors from adenylyl cyclase3 by an increased expression of β-adrenergic receptor kinase (βARK-1).4 Investigations of transgenic mice have shown that β-adrenergic signaling can be “resensitized” in the long term by overexpressing the βARK inhibitor “βARKmini.”16,17 In a transgenic mouse model of heart failure due to disruption of actin filaments (MLP_{2}/_{2} mice), overexpression of βARKmini pre­vented the development of heart failure, whereas overexpression of β2-adrenergic receptors even precipitated it.18 Because of these diverging results for proteins that all increase intracellular cAMP levels, specific transgenic interventions in the G protein–related messenger system might produce beneficial effects in the long run in vivo, despite the well-known failure of some cAMP-raising pharmacological agents to produce better long-term outcomes.19

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**Figure 5.** Hemodynamic data of rats that had received a direct myocardial injection of adenovirus coding for β-Gal (Ad–β-Gal, A) or V2Rs (Ad–V2R, B). Effect of a systemic bolus injection of 10 or 100 pmol DDAVP on echocardiographic fractional shortening (A) and on global dP/dt_{max} in the left ventricle (B). Respective dP/dt min values (in mm Hg/s) were \(-1411±60,\) \(-1415±53,\) and \(-1293±58,\) \(-1320±46,\) and \(-1323±143\) in B.
Our report complements these findings, inasmuch as it shows that somatic gene transfer of a heterologous protein that stimulates endogenous adenyl cyclase can also increase cardiac contractility.

Limitations of the Study

Intermittent as opposed to continuous cAMP stimulation via heterologous rV2Rs might therefore be superior to some previous approaches, although this assumption has to be proved in an ongoing study on the long-term effects of V2R overexpression. We have shown in the present study that ectopic expression of the V2R can be a sufficient, alternative method to bypass the altered β-adrenergic signaling pathway in the heart. Moreover, our previous study has shown that rV2Rs expressed in cardiomyocytes are not subject to downregulation, so that they can maintain their function even after prolonged agonist exposure. Whether this is also true for the long-term overexpression of V2Rs in vivo remains to be determined.

Note Added in Proof

During the review process of the present article, successful somatic gene transfer of β2-adrenergic receptors to rabbit hearts has been reported by Maurice et al (J Clin Invest. 1999;104:21–29).

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