Cardiac Overexpression of the Norepinephrine Transporter Uptake-1 Results in Marked Improvement of Heart Failure

Götz Münch, Kai Rosport, Andreas Bültmann, Christine Baumgartner, Zhongmin Li, Lien Laacke, Martin Ungerer

Abstract—A hyperadrenergic state is one of the key features of human and experimental heart failure. Decreased densities and activities of the presynaptic neuronal norepinephrine (NE) transporter uptake-1 occur both in patients and animal models. It is currently unclear to what extent the reduction of uptake-1 contributes to the deterioration of heart failure. Therefore, we investigated the effects of myocardial overexpression of uptake-1 in both nonfailing rabbit hearts and in an animal model of heart failure. Heart failure was induced in rabbits by rapid ventricular pacing. Adenoviral gene transfer was used to overexpress uptake-1 in the myocardium. Uptake-1 overexpression led to increased NE uptake capacity into the myocardium. In contrast, systemic plasma NE levels in uptake-1-overexpressing failing rabbits (uptake-1–CHF) did not differ from controls. Downregulation of SERCA-2 and β-adrenergic receptors in the failing myocardium was significantly reversed after uptake-1 overexpression. Uptake-1 overexpression significantly improved left ventricular (LV) diameters (LV end-diastolic diameter: in GCP-overexpressing failing rabbits (GFP-CHF), 17.4±0.4 mm; in uptake-1–CHF rabbits, 15.6±0.6 mm) and systolic contractility (fractional shortening: GFP-CHF, 20.7±0.6%; uptake-1–CHF, 27.3±0.7%), as assessed by echocardiography at the end of the heart failure protocol. Intraventricular tip catheter measurements revealed enhanced contractile reserve (dP/dt max with isoproterenol 1.0 μg/kg: GFP-CHF, 6964±230 mm Hg/sec; uptake-1–CHF, 7660±315 mm Hg/sec) and LV relaxation (dP/dt min with isoproterenol 1.0 μg/kg: GFP-CHF: −3960±260 mm Hg/sec; uptake-1–CHF, −4910±490 mm Hg/sec). End-diastolic filling pressures (GFP-CHF, 8.5±1.2 mm Hg; uptake-1–CHF, 5.6±0.7 mm Hg) tended to be lower in uptake-1 overexpressing animals. In summary, local overexpression of uptake-1 in the myocardium results in marked structural and functional improvement of heart failure, thus underlining the importance of uptake-1 as a key protein in heart failure.

(Circ Res. 2005;97:928-936.)

Key Words: heart failure ■ catecholamines ■ norepinephrine transporter ■ gene transfer ■ animal heart failure model

Heart failure is characterized by a marked increase in sympathoadrenergic activity. Increased systemic levels of norepinephrine (NE), which correlate to the prognosis of the patients,1 and increased local cardiac spillover of catecholamines are 2 key elements observed in patients with heart failure. Cardiac NE spillover is the net result of NE release from nerve terminals and clearance of synaptic NE mainly caused by reuptake via the uptake carriers. A reduction of cardiac uptake-1 density or function has been documented in tissue samples of both human patients and animal models of the disease and might contribute to this increase in NE spillover.2–4 A decrease of myocardial NE uptake-1 has also been found to correlate with age in humans.5 Clinical studies of patients with terminal heart failure have shown an increased cardiac NE turnover at rest,6,7 as well as a failure to reach the normal maximum spillover during exercise.8 These studies have also provided evidence that presynaptic NE release is increased and reuptake of catecholamines is relatively reduced in these patients in vivo.6–7 The rate-limiting step for cardiac catecholamine extraction in patients with heart failure, however, is the function of uptake-1. A reduced and heterogeneous uptake-1 activity has been documented in the failing human heart in vivo by scintigraphic imaging using the tracer meta–iodobenzyl guanidine in a single-photon emission computed tomography technology9,10 or C11-Hydroxyephedrine for positron emission tomography.11,12 The marked increase in NE release has been widely thought responsible for various postsynaptic alterations in the failing heart. Blunted responsiveness of the β-adrenergic receptor system, with downregulation of β-adrenergic receptors, increased activity of G protein–coupled receptor kinase 1, decreased stimulability of adenylyl cyclase,13 and various alterations of calcium cycling are key features of autonomic nervous system dysregulation.

An animal model of heart failure in which alterations of uptake-1 have so far been extensively characterized is rapid
ventricular pacing in rabbits. In this model, marked down-regulation of NE uptake function and uptake-1 density already occur after 2 weeks of pacing. Treatment with the monoamine oxidase type B inhibitor seleagine or with angiotensin-converting enzyme inhibitors resulted in a reversion of these alterations and in a concomitant attenuation of the hemodynamic alterations caused by rapid pacing. It is currently unclear, however, whether down-regulation and decreased activity of uptake-1 in heart failure are coincidental or causally related. We therefore sought to investigate whether a reversal of decreased uptake-1 function would have a beneficial effect on heart failure and studied cardiac overexpression of uptake-1 for its effects on contractility and the development of heart failure in this animal model.

Materials and Methods

Construction and Purification of Recombinant Adenovirus

The present investigation was done with the cDNA for the bovine uptake-1 ligated into adenoviral shuttle vectors as described before. Recombinant (E1/E3-deficient) adenoviruses were generated, expressing C-terminally flag-tagged uptake-1 and green fluorescence protein (GFP) under control of 2 independent cytomegalovirus promoters. As a control, Ad-GFP without further transgenes was used. Large virus stocks were prepared as described previously.

Norepinephrine Uptake Measurements in Isolated Cells

PC-12 cells were used for measurement of endogenous NE transport activity. HeLa cells were infected with either Ad-GFP or Ad-uptake-1-GFP (multiplicity of infection of 10) as described in detail in the online-only data supplement available at http://circres.ahajournals.org.

Model of Heart Failure

Pacemakers from Vitatron were implanted into New Zealand White rabbits (weight 3.6–0.3 Kg; from Assam, Aretshied, Germany). One week afterward, rapid pacing was initiated at 320 bpm. Under this protocol, a tachycardia-induced heart failure (HF) develops reproducibly over 1 week. Pacing was then continued at 360 bpm, which predictably led to a further deterioration of the heart. Nonfailing rabbits were sham-operated, but rapid pacing was not initiated. The project was performed according to animal protection guidelines and approved by the respective supervising authority.

Adenoviral Gene Transfer to Rabbit Myocardium

Both nonfailing rabbits and rabbits with heart failure attributable to rapid LV pacing received adenoviral gene transfer (4x10^10 pfu) to the myocardium by aortic and pulmonary crossclamping as described in detail in the data supplement. Adenovirus was administered at day 0 and directly after the basal echocardiography measurement.

Measurement of Cardiac Function and LV Hemodynamics

Left ventricular contractility and dimensions were measured by echocardiography in all rabbits at baseline and 1 and 2 weeks after gene transfer. For final LV catheterization, tip catheter measurements were performed 14 days after gene transfer both in nonfailing and failing rabbits as described in the data supplement.

Immunoblotting

Tissues or cells were homogenized and used for immunoblotting. Expression of uptake-1, SERCA-2, phospholamban, sodium/calcium exchanger, calcequestrin, and β1-adrenoceptors were detected with specific antibodies.

Immunohistochemistry of Recombinant Uptake-1

Myocardial slices from rabbit hearts harvested 2 weeks after gene transfer were frozen. Detection of transgenic uptake-1 expression was performed with an antibody raised against flag tag (which is fused to the recombinant uptake 1 protein; ANTI-FLAG M2 antibody; Sigma). Antibody binding was visualized with an avidin/biotinylated glucose oxidase system according to the manufacturer’s instructions (Vectastain ABC-GO Kit, Vector Laboratories Inc).

Norepinephrine Uptake Measurements in Tissue Slices

Tissue slices were prepared from the septal, anterior, and inferior left ventricular myocardium. The tissues were prepared directly after excision of the heart as previously described in detail. Specific uptake was defined as total uptake (37°C) minus nonspecific uptake (4°C). All incubations were performed in triplicate. After careful washing, radioactivity in the tissue slices was determined by β-counting.

Measurement of Cardiac NE Content

For NE determinations, tissue samples were homogenized (Ulturarax) in 0.1 mol/L HCl. After centrifugation at 2000g (4°C, 10 minutes), the protein content of the supernatant was determined by the DC method (BioRad Laboratories) and NE was extracted using a catecholamine extraction kit (Chromsystems) and measured by high performance liquid chromatography with electrochemical detection.

Measurement of NE Serum Levels

Blood samples were taken from the animals before gene transfer and 2 weeks after gene transfer. After centrifugation at 2000g for 10 minutes, the serum was stored at −80°C for NE determination.

Data Analysis

The data are summarized from n=8 animals for each group. All data are expressed as mean±standard error of the means (SEM). For statistical analysis, analysis of variance (ANOVA) for independent measurements was used. For all analyses, a value of P<0.05 was considered to be statistically significant.

Results

Effect on NE Uptake

Infection of cultured HeLa cells with Ad-uptake-1-GFP (which codes both for the reporter gene and for the gene of interest) led to markedly enhanced extraction and uptake of NE from the culture medium. The addition of up to 50 μmol/L NE to the culture medium resulted in the intracellular accumulation of increasing amounts of NE, with a clear saturation (Figure 1A). The kinetic constants (mean K_M-values of 620 nmol/L, insert to Figure 1A) corresponded well to those observed in uptake-1–containing PC-12 cells, which were investigated under the same conditions. K_M values were also comparable to previously published values of uptake-1. NE uptake into Ad-uptake-1-GFP-infected HeLa cells showed a half-maximal time in accordance with the literature. It was almost completely and potently inhibited by desipramine at a Ki of ≈7 nmol/L (Figure 1B), whereas desipramine only partially inhibited NE uptake into PC-12 cells. In contrast, no extraction was observed with Ad-GFP-infected control cells.

In Vivo Adenoviral Delivery of Uptake-1 to Failing Hearts

Overexpression of uptake-1 was investigated by studying the coexpression of green fluorescent protein (GFP) in the hearts
after in vivo gene transfer and compared with the expression of GFP in the control group. For this purpose, fresh sections were cut from the hearts treated by gene transfer in vivo. Figure 2A shows an example of a macroscopic slice of a rabbit heart infected with Ad-uptake-1-GFP. Staining with a specific anti-GFP antibody shows GFP coexpression throughout the left ventricle (lower panel), whereas no signal occurred in a nontransfected control heart (upper panel).

Transgene Expression Assessed by Western Blotting
Western blotting documented that specific expression of uptake-1 was detectable with an approximate size of 100 kDa (see figure 2B) in accordance with Bauman and coworkers (please see20). Uptake-1 expression in the myocardium was significantly increased in rabbits after gene transfer with Ad-uptake-1-GFP (see figure 2D).

Recombinant Uptake-1 Transgene Expression
Hearts and other organs were harvested from rabbits 14 days after myocardial gene transfer via aortic cross clamping. Immunohistochemistry of the recombinant uptake 1 in myocardial slices from rabbit hearts showed marked expression of the recombinant uptake-1 in cardiomyocytes, but also in extracellular matrix, connective tissue, and the cells therein, such as fibrocytes and possibly sympathetic nerve endings (Figure 2C). Postmortem investigation of recombinant uptake-1 expression in other organs from identical rabbits revealed positive immunofluorescence signals in the spleen, weak signals in the liver, and virtually no fluorescence in the lungs, kidneys, and calf muscles of these rabbits (data not shown).

Expression of Other Cardiac Proteins
The expression of key proteins regulating the intracellular myocardial calcium homeostasis such as SERCA-2, sodium/calcium exchanger, and phospholamban and of β1-adrenoceptors were assessed in rabbit myocardium. SERCA-2 expression was significantly decreased in failing myocardium compared with nonfailing control rabbit myocardium. Similarly, expression of β1-adrenoceptors was significantly downregulated in failing myocardium. After overexpression of uptake-1 in the rabbit heart failure model, this downregulation of SERCA-2 and β1-adrenoceptors was reversed, and their expression reached levels similar to those seen in nonfailing myocardium (Figure 2D).

Heart Failure
Rapid pacing was used to induce heart failure in rabbits. At the end of the pacing protocol, rabbits developed clinical signs of heart failure such as pleural effusions (nonfailing...
rabbits $0 \pm 0 \text{ mL}$; failing rabbits $4.1 \pm 0.4 \text{ mL}$) and increased liver weight (nonfailing rabbits $65 \pm 8 \text{ g}$; failing rabbits $104 \pm 8 \text{ g}$). The average $+\text{dP/dt}_{\text{max}}$ value in failing hearts was $1800 \pm 170 \text{ mm Hg/sec}$ (versus $2900 \pm 470 \text{ mm Hg/sec}$ in healthy controls; $P<0.05$), and left ventricular end-diastolic pressure (LVEDP) increased from $3.5 \pm 0.6 \text{ mm Hg}$ to $8.5 \pm 1.4 \text{ (P}<0.05)$.

**Cardiac NE Uptake Capacity**
At the end of the pacing period, the biochemical effects of gene transfer of Ad-uptake-1-GFP were compared with those of Ad-GFP. Heart failure led to a significant decrease in cardiac NE extraction throughout several regions of the myocardium (Figure 3). Figure 3 shows the results after incubation with $38 \text{ nmol/L}$ NE; incubation with $19 \text{ nmol/L}$ gave corroborating results (although lower absolute values; data not shown). Overexpression of uptake-1 led to significantly increased cardiac NE extraction capacity via the uptake-1 carrier in rabbits with heart failure compared with failing rabbit hearts expressing only GFP (Figure 3). The values in the failing uptake-1 group almost reached those of healthy animals (Figure 3).

**Cardiac NE Content**
In myocardium from uptake-1–overexpressing failing rabbit hearts, local NE content was significantly increased compared with that of myocardium of failing rabbit hearts expressing only GFP ($9.4 \pm 0.4 \text{ ng/mg tissue}$ versus $7.5 \pm 0.13 \text{ ng/mg}$, $P<0.01$).

**Effect on Systemic Plasma NE Levels**
Plasma NE levels in uptake-1–overexpressing nonfailing rabbit hearts (values at the end of the experiment: $310 \pm 20 \text{ pg/mL}$) were not different compared with values in nonfailing control rabbit hearts ($380 \pm 30 \text{ pg/mL}$). Plasma NE levels in uptake-1–overexpressing rabbits with heart failure (values at the end of the experiment: $577 \pm 40 \text{ pg/mL}$) were not decreased compared with GFP-expressing rabbits with heart failure ($452 \pm 90 \text{ pg/mL}$).

**Weight Gain**
Uptake-1 overexpression resulted in decreased body weight gain during tachycardic pacing (body weight difference between start of pacing and end of experiment in GFP-expressing failing rabbits: $+108 \pm 58 \text{ g}$; in uptake-1/GFP-expressing failing rabbits: $-22 \pm 40 \text{ g}$).

**Improvement of LV Dysfunction in Pacing-Induced Heart Failure**
In both nonfailing rabbits and failing rabbits with rapid ventricular pacing, the effects of gene transfer of Ad-uptake-1-GFP on cardiac function were compared with those of Ad-GFP, as quantified by echocardiography 7 days and 14 days after adenoviral gene delivery. A final hemodynamic measurement with tip catheterization 14 days after adenoviral cross-clamping served to complement this information.

In nonfailing rabbits, left ventricular fractional shortening (FS) determined by echocardiography was not significantly different after gene transfer with Ad-uptake-1-GFP compared...
with Ad-GFP (after 1 week: Ad-GFP 38.0 ± 1.0% versus Ad-uptake-1-GFP 36.2 ± 1.2%; 2 weeks after gene transfer Ad-GFP 32.7 ± 1.3% versus Ad-uptake-1-GFP 32.9 ± 1.4%). The mean wall thickness also did not differ significantly in the Ad-uptake-1-GFP–infected nonfailing rabbits (Ad-GFP 0.29 ± 0.01 cm versus Ad-uptake-1-GFP 0.30 ± 0.02 cm; P < 0.01). Figure 4 shows that the left ventricular end-diastolic dimensions in rabbits with rapid LV pacing, assessed by serial echocardiography, were more dilated in the Ad-GFP–infected than in the Ad-uptake-1-GFP–infected group at the end of the experiment (Figure 4). LV FS in rabbits with rapid LV pacing was significantly improved in the uptake-1–expressing group compared with the Ad-GFP–infected group, after both 1 week and 2 weeks of rapid pacing (Figure 5). In contrast, the mean wall thickness did not differ between the 2 groups (data not shown).

At the end of the experiment, intraventricular tip catheterization was used to determine hemodynamic parameters. In uptake-1–expressing nonfailing rabbits, the basal first derivative of LV pressure (dP/dt max) was not different from the values in Ad-GFP–infected rabbits (Ad-GFP 2855 ± 472 mm Hg/sec versus Ad-uptake-1-GFP 2676 ± 209 mm Hg/sec). The dP/dt max on isoproterenol stimulation was also not significantly different between groups at 0.5 μg/kg (Ad-GFP 7670 ± 1225 mm Hg/sec versus Ad-uptake-1-GFP 8362 ± 946 mm Hg/sec) or at other isoproterenol doses. The left ventricular end-diastolic filling pressure 14 days after gene transfer also did not differ significantly between both groups of healthy rabbits (Ad-GFP 3.5 ± 0.6 mm Hg; Ad-uptake-1-GFP 2.2 ± 0.8 mm Hg).

Animals with heart failure due to rapid LV pacing whose hearts expressed uptake-1 showed a trend toward lower LV filling pressures compared with the group expressing GFP only (Figure 6), although this trend did not reach statistical significance. Left ventricular pressure was significantly increased in Ad-uptake-1-GFP–infected failing rabbits (Ad-GFP 60 ± 4 mm Hg versus Ad-uptake-1-GFP 71 ± 3 mm Hg). Figure 7 shows that in the uptake-1–expressing group of failing rabbits, the dP/dt max in response to isoproterenol was significantly higher than in the Ad-GFP–infected control group, indicating an improvement in contractile reserve. The minimum velocity of shortening (dP/dt min), which corresponds to LV relaxation, was also significantly improved with uptake-1 overexpression in the failing rabbits (Figure 8).

**Discussion**

The present study shows that in vivo gene transfer of the neuronal catecholamine transporter uptake-1 to the myocardium results in a clear improvement of cardiac function in
rabbits with heart failure. These results suggest that increased local clearance of catecholamines, and above all of NE, should confer a marked therapeutic benefit in heart failure.

Decreased cardiac uptake-1 function in heart failure has been documented in a number of studies.6–10 The question of whether this finding reflects a general decrease of cardiac sympathoadrenergic neurons or a selective decrease of NE transporter proteins on these cells has not been fully clarified. Although some early studies claimed a decreased cardiac atrial neuron number in heart failure,21 recent evidence supports the notion that the transporters are specifically downregulated and other neuronal functions are intact. Release of NE from cardiac sympathetic nerve endings is clearly increased in heart failure, and elevated NE levels have been documented in the coronary sinus of these patients.6

It has been known for a long time that congestive heart failure is associated with chronic activation of the sympathoadrenergic system. Sympathetic activation is one of the most efficient and useful cardiac regulation mechanisms for acute adaptation to increased cardiac demand. The long-term effects of sympathetic activation, however, seem to be detrimental. The introduction of β-adrenoreceptor blockers has been one of the most significant milestones in the therapy of heart failure and is thought to act by reducing chronic sympathetic stimulation.22 Additionally, several clinical trials studied sympatholytic agents with the aim of reducing sympathoadrenergic activity. Unfortunately, however, a trial studying the imidazoline or α2 adrenergic receptor agonist moxonidine in heart failure had to be terminated early (MOXCON trial), as an increased mortality was observed in the treatment group.23 Moxonidine powerfully lowers systemic NE concentrations. The failure of the trial was ascribed to the short-term side effects of such a general sympatholysis.23 Similarly, bucindolol, a β-adrenoreceptor blocker with a strong sympatholytic component, increased the risk of death, especially in the patient group that experienced the strongest decline in plasma NE levels.24

Hence, it was our hypothesis that in contrast to a general reduction of systemic adrenergic activity, a specific removal of locally released cardiac NE might produce different, clearly more positive effects in heart failure. We therefore wished to test this theory by studying an approach that allows for a specific reduction of intracardiac NE concentrations without affecting the systemic release and the systemic

Left Ventricular Enddiastolic Pressure (LVEDP; Tip Catheter)
effects of catecholamines. The approach was to use local gene transfer, which is largely specific for the myocardium. Localization of the recombinant uptake-1 protein in the myocardium was verified by specific immunohistology of the protein. In other organs, the expression of recombinant uptake-1 after aortic cross-clamping was weak or not detectable. Accordingly, local myocardial NE uptake was significantly increased in failing myocardium with uptake-1 overexpression. Moreover, local NE tissue content in the myocardium with uptake-1 overexpression was also significantly increased. On the other hand, systemic sympatho-adrenergic function was unchanged, as demonstrated by unaltered NE serum levels. A previous study in a rat heart failure model linked neurohumoral activation with the decrease of NE transporter density and postsynaptic ß-adrenoceptor downregulation, as typically found in heart failure patients. Moreover, increased sympathetic activity also affects intracellular calcium cycling by impairment of SERCA-2 function. Thus, the observed beneficial effects of uptake-1 overexpression were most probably due to a protection of cardiomyocytes from the deleterious effects of chronic catecholamine overstimulation. In fact, uptake-1 overexpression completely reversed ß1-adrenoceptor downregulation, which also occurred in the present rapid pacing-induced heart failure in rabbits, up to levels that occur in nonfailing rabbit myocardium. Moreover, decreased SERCA-2 expression, which is one of the most relevant alterations that explains the impaired intracellular calcium transport capacity in cardiomyocytes, was largely ameliorated in failing hearts after uptake-1 overexpression.

The finding of improved contractility after gene transfer of Ad-uptake-1-GFP during heart failure in vivo corresponds to the improvement of LV function measured in knockout mice for dopamine beta hydroxylase, a key enzyme in NE synthesis. These mice were protected against the development of cardiac hypertrophy after aortic banding. Moreover,
an investigation of α2 adrenergic receptor knockout mice lacking the feedback inhibition for NE release underlined the importance of anti-sympathetic mechanisms in heart failure.28

Our study now shows for the first time that a genetic intervention aiming at lowering local synaptic NE concentrations also attenuates a dilated heart failure phenotype. Overexpression of uptake-1 increased the cardiac NE uptake. This increased uptake-1 activity led to a local improvement of the cardiac hyperadrenergic state observed in heart failure. Consequently, we observed a clear improvement of heart failure and reversal of key features of chronic catecholamine overstimulation, such as β1-adrenoceptor downregulation and decreased expression of intracellular calcium-handling proteins such as SERCA-2. Therefore, loss of uptake-1 function in heart failure seems to be a pivotal problem that could well be the cause of a variety of postsynaptic alterations observed in heart failure. On the other hand, we found indirect evidence in support of increased neuronal reuptake and vesicular storage of NE in sympathetic nerve endings in the myocardium after Ad-uptake-1-GFP gene transfer. First, myocardial NE uptake was significantly increased by uptake-1 overexpression. Secondly, NE content in myocardial homogenates was significantly higher compared with failing GFP controls. The high pressure liquid chromatography-based NE detection system used in this investigation is highly specific for NE and shows no cross-reactivity with NE metabolites. Therefore, NE reuptake by recombinant uptake-1 must be at least partially neuronal reuptake of NE and vesicular storage in the sympathetic nerve ending. NE uptake into the nerve terminal without vesicular storage and also uptake into nonneuronal tissue after uptake-1 overexpression would, however, result in rapid degradation of NE either directly by MAO or indirectly via the extra-neuronal enzyme catechol-o-methyltransferase. Thus, it can be assumed that recombinant uptake-1 proteins after Ad-uptake-1-GFP infection are, at least partially, located in sympathetic nerve endings. The immunohistology of uptake-1 in the myocardium revealed overexpression in both cardiomyocytes and the extracellular matrix, such as fibrocytes and possibly also nerve endings. The data on NE tissue content strongly suggest that recombinant uptake-1 is also localized in sympathetic nerve terminals, resulting in physiologically correct NE reuptake into synaptic storage vesicles.

It has to be stated that the present heart failure model—the combination of tachycardia-induced heart failure and adenoviral-induced myocarditis—leads to an intermediate degree of heart failure. Kawai et al16 showed higher LVEDP in rabbits with pacing-induced heart failure than our study, but all other hemodynamic parameters could be very well reproduced (eg, the decrease in dP/dt max was quite comparable between our study and that of Kawai et al). Moreover, measurement of contractility by echocardiography revealed quite similar values for fractional shortening in our heart failure model (Figure 5) compared with the published FS data from Kawai’s group. Additionally, other features of heart failure, such as water retention and congestion due to LV failure, could be documented, indicating clear clinical signs of moderate to severe heart failure in these animals. Differences in anesthesia could partially account for the discrepancies of pressure measurements. Whereas Kawai et al and others used ketamine/midazolam, we used fentanyl/propofol, as the animals tolerated fentanyl/propofol much better and could be much better controlled during anesthesia.

In contrast, overexpression of uptake-1 with the same gene transfer protocol has no significant effect on myocardial contractility and hemodynamics in nonfailing rabbits. Thus, nonspecific effects of adenoviral delivery can be largely excluded. The alterations during heart failure, such as uptake-1 downregulation and increased local NE spillover, seem to be a prerequisite for the beneficial effects of uptake-1 overexpression. Therefore, there seems to be a strong link between uptake-1 expression and heart failure, as uptake-1 overexpression has specific effects in heart failure.

In conclusion, local overexpression of uptake-1 in the myocardium results in significant improvement of heart failure. The findings stress the importance of uptake-1 as a key protein in heart failure. Thus, interventions directed at increasing the clearance of NE from the cardiac synaptic cleft should present a novel and promising concept for the treatment of heart failure.

Limitations of the Study

The present study clearly shows a compensatory role of uptake-1 overexpression in the myocardium of failing rabbits. The present data, however, do not prove unequivocally that there is a primary and causal relationship between reduced uptake-1 function and the development or progression of heart failure. Moreover, some parameters of heart failure were only modestly improved by uptake-1 overexpression. As we cannot exactly localize the overexpression of uptake-1 to a specific cell type in the myocardium, uptake-1 overexpression after gene transfer will not always lead to a functional recycling of NE. Hence, the present results provide strong hints that local clearance of NE should be beneficial in heart failure. We could not unequivocally show that an increase in myocardial neuronal uptake-1 function must also be beneficial, as recycling and even increased NE release could also result from such an intervention. Thus, further studies with cell-specific overexpression of uptake-1 to neuronal or nonneuronal cells might further help to elucidate the role of uptake-1 in heart failure. Moreover, the causal role of uptake-1 might be clarified further in future experiments under pathophysiological conditions in an overexpression model for uptake-1 on a genetic background where uptake-1 is absent.

Acknowledgments

This study was supported by a grant of the Bayerische Forschungstiftung (project # 446/01).

References


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Circ Res. 2005;97:928-936; originally published online September 15, 2005;
doi: 10.1161/01.RES.0000186685.46829.E5

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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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:
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Norepinephrine uptake measurements in isolated cells: PC-12 cells were used for measurement of endogenous NE-transport activity. HeLa cells were infected with either Ad-GFP or Ad-uptake-1-GFP (moi of 10). One day thereafter, the cells were preincubated at 37°C for 20 min with buffer A (125 mmol/L NaCl, 4.8 mmol/L KCl, 1.2 mmol/L CaCl₂, 1.2 mmol/L KH₂PO₄, 1.2 mmol/L MgSO₄, 25 mmol/L HEPES, pH 7.4, 5.6 mmol/L D(+)glucose, 1 mmol/L L(+)ascorbic acid, 10 µmol/L pargyline) and then incubated for 15 min with increasing concentrations of NE (norepinephrine-bitartrate salt; Sigma, A9512) in buffer A. The incubation was stopped by washing the cells four times with ice-cold buffer A. The cells were solubilized by adding 0.1 % (v/v) Triton X-100 in 5 mmol/L Tris/HCl, pH 7.5. The lysate was clarified by centrifugation (20000 rpm, 5 min, 4°C). For determination of endogenous NE uptake activity, undifferentiated PC-12 cells were incubated with similar NE concentrations for 15 min. Unspecific NE uptake was measured in the presence of desipramine (10 µmol/L). NE concentrations were determined in this extract by high performance liquid chromatography with electrochemical detection.

Adenoviral Gene Transfer to Rabbit Myocardium: Both non-failing and failing rabbits due to rapid LV pacing received adenoviral gene transfer (4x10¹⁰ pfu) to the myocardium by aortic and pulmonary crossclamping.

In the two groups, 8 rabbits each received Ad-GFP for control or Ad-uptake-1-GFP as the gene of interest. For all interventions, the rabbits were anesthetized with FentanylR (0.01 mg/kg) and PropofolR (2%; 80 mg/kg/h). The animals were continuously ventilated after intratracheal intubation. ECG and pulse oximetry was monitored. After accessing the left carotid artery, an arterial sheath was inserted and a 3F catheter was introduced and placed in the aortic bulb in front of the openings of the coronary arteries. Lateral thoracotomy between 2nd and 3rd rib was performed and the pericardium was removed. Aorta and pulmonary arteries
were ligated with sutures to stop blood flow. Simultaneously, the virus solution containing $4 \times 10^{10}$ pfu (either Ad-GFP or Ad-uptake-1-GFP) was injected into the coronaries under X-ray control while aorta and pulmonary arteries were cross-clamped. Thereafter blood flow was restored by removal of sutures. After a stabilization period to allow normalization of haemodynamic conditions, the catheter was removed and thorax closed by sutures. Postoperative analgesia was performed with Temgesic™ (2 x 0.01 mg/kg/d) and Rimadyl™ (4 mg/kg/d). The efficacy of gene transfer was assessed in the hearts after the end of the experiments by investigating transverse freeze-cut sections for expression of GFP by fluorescence microscopy under UV long-wave illumination. Gene transfer led to reproducible transgene expression in ~50% of cardiomyocytes. Moreover, transgene expression was also verified two weeks after adenoviral gene transfer by specific immunohistochemical detection of the recombinant uptake-1 protein in myocardial slices. Additionally, functionally effective gene transfer was studied by determination of specific uptake transport capacity for NE in the myocardium after gene transfer. The pacing protocol was started 6 hours after gene transfer for the heart failure group with no pacing in the non-failing rabbits.

**Measurement of Cardiac Function and LV hemodynamics:** Left ventricular contractility and dimensions were measured by echocardiography in all rabbits at baseline, and one and two weeks after gene transfer. Echocardiography was performed using a 7.5 MHz probe (Hewlett Packard, Sonos 1000) after the induction of anaesthesia with Fentanyl™ (0.01 mg/kg) and Propofol™ (2%; 80 mg/kg/h). For echocardiography, the 7.5 MHz probe was fixed on a tripod. Standard sections were recorded. Reproducibility of measurements was secured by triplicate measurements and repeated, serial pilot measurements in control animals without any intervention. Both, M mode and B mode measurements were carried out. ECG was monitored continuously during the measurement.
For final LV catheterization 14 days after the adenoviral gene delivery, a 3F micromanometer-tipped catheter (Millar) connected to a differentiating device was placed in the left ventricle via a sheath in the carotid artery. After definition of basal contractility and left ventricular pressure, 200 µL of NaCl (0.9%) was injected as a negative control. Isoproterenol was infused intravenously at increasing doses. After a 20 min equilibration period, tip catheter measurements were carried out with triplicate measurements.