24-h Langendorff-perfused neonatal rat heart used to study the impact of adenoviral gene transfer


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Am J Physiol Heart Circ Physiol 285: H907–H914, 2003. First published March 27, 2003; 10.1152/ajpheart.00856.2002.—The human genome project has increased the demand for simple experimental systems that allow the impact of gene manipulations to be studied under controlled in vivo conditions. We hypothesized that, in contrast to adult hearts, neonatal hearts allow long-term perfusion and efficient gene transfer ex vivo. A Langendorff perfusion system was modified to allow perfusion for >24 h with particular emphasis on uncompromised contractile activity, sterility, online measurement of force of contraction, inotropic response to β-adrenergic stimulation, and efficient gene transfer. The hearts were perfused with serum-free medium (D-MEM + medium 199, 4 + 1) supplemented with hydrocortisone, triiodothyronine, ascorbic acid, insulin, pyruvate, L-carnitine, creatine, taurine, L-glutamine, mannitol, and antibiotics recirculating (500 ml/2 hearts) at 1 ml/min. Hearts from 2 day-old rats beat constantly at 135–155 beats/min and developed active force of 1–2 mN. During 24 h of perfusion, twitch tension increased to ~165% of initial values (P < 0.05), whereas the inotropic response to isoprenaline remained constant. A decrease in total protein content of 10% and histological examination indicated moderate edema, but actin and calasequestrin concentration remained unchanged and perfusion pressure remained constant at 7–11 mmHg. Perfusion with a LacZ-encoding adenovirus at 3 × 10⁸ active virus particles yielded homogeneous transfection of ~80% throughout the heart and did not affect heart rate, force of contraction, or response to isoprenaline compared with uninfected controls (n = 7 each). Taken together, the 24-h Langendorff-perfused neonatal rat heart is a relatively simple, inexpensive, and robust new heart model that appears feasible as a test bed for functional genomics.

In cardiovascular research attempts have been made to create easier and more efficient experimental models that are less prone to compensational responses but yet provide the necessary complex cardiac context. Examples are cultured neonatal rat cardiac myocytes (11), freshly isolated adult cardiac myocytes (5, 6, 16), isolated trabeculae of adenovirus-perfused rabbit hearts (17), injection of adenovirus into neonatal rat hearts in vivo (4), or adenovirus infusion into adult rat hearts in vivo (12). Cells in culture are easily transfected but are either immature or prone to cell isolation and dedifferentiation artifacts. In addition, measurement of force of contraction is indirect at best. More complex systems likely better reflect the physiological situation and allow force measurement, but they require high virus titers, i.e., are expensive and time consuming, require higher technical demands, and have not yet been shown to be applicable as a routine method. Recently, we have introduced the method of engineered heart tissue that may serve as an intermediate between cell culture and animal model by allowing efficient adenoviral gene transfer and measurement of isometric force

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of contraction (9, 23). However, adenoviral gene toxicity appears to limit its usefulness as a routine method (7). Thus none of the existing systems appears to be a perfect test bed for adenoviral gene transfer. The classic Langendorff heart may be ideally suited for this purpose because it allows measurement of complex cardiac function under relatively physiological conditions. Yet, in hearts from adult animals of various species development of interstitial edema limits perfusion time to maximally 6–8 h, and attempts to circumvent this problem by modifying the perfusion conditions have failed (21). A recent review describes that hypothermic arrest periods cause tissue preservation up to 24 h and longer (20), but no attempts have been made to use this model for genetic testing. We hypothesized that neonatal rat hearts are less sensitive to the artificial conditions of in vitro perfusion with colloidal solutions, partly because they are very hypoxia/ischemia resistant (1, 10) and have a less developed vascular system and therefore may serve as a suitable, improved model to study effects of adenoviral gene transfer.

METHODS

All procedures were approved by the local animal protection authority (TS-00/20 Pharma) and conformed to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Publication 85-23, Revised 1985).

Animals. Langendorff perfusion setup, and medium. A total number of 710 Wistar pups (days 0–7) was used. The mean weight was 7.9 g. Animals received an intraperitoneal injection of heparin (500 units) 10–20 min before being euthanized. After cervical dislocation, hearts were rapidly excised (mean time to perfusion 2.5 min) and mounted on a Langendorff-perfused heart; SW, steal wire; SG, force transducer with strain gauges; PC, personal computer. B: photograph of a detail of the perfusion setup. BT, bubble trap; LH, Langendorff-perfused heart; SW, steal wire connecting the heart with the force transducer; SG, force transducer with strain gauges.

Fig. 1. Experimental setup for 24-h Langendorff perfusion of neonatal rat hearts. A: schematic diagram of the setup. 1 and 2, carbogen-gassed, water-jacketed medium reservoirs; 3, one-way sterile filter; 4, pump; 5, water-jacketed organ bath; P, perfusion pressure transducer; SG, force transducer with strain gauges; PC, personal computer.
of Microbiology, University of Erlangen-Nuremberg. This evaluation showed no sign of bacterial or fungal growth.

Heart wet-to-dry weight and protein determination. To determine heart-to-body weight ratios, animals were weighed, and hearts including atria were excised and gently, but thoroughly dabbed between two layers of paper towels either before or after the 24-h perfusion, weighed, and then freeze-dried (24 h) in preweighed Eppendorf cups. To determine protein content using the Bradford method (Bio-Rad), hearts either native or after the 24-h perfusion were homogenized in lysis buffer (Tris, pH 7.5, 25 mmol/l, SDS 5%), further diluted with water, and measured with IgG as standard.

Adenovirus production and titering. The adenovirus coding for bacterial β-galactosidase with a nuclear localization signal (Ad5LacZ) was a kind gift from Y. Fromes, Paris, France. It was propagated in HEK 293 cells and titered on neonatal rat cardiac myocytes as described previously (23). Importantly, the number of biologically active virus (bav) was defined as the virus number necessary to positively transfect (stain blue) a given number of neonatal rat cardiac myocytes in culture. For example, 1 × 10⁶ bav was just sufficient to transfect 1×10⁶ cardiac myocytes or 50% of 2×10⁶ cardiac myocytes.

Adenoviral gene transfer: standard experiment. The hearts were infected with Ad5LacZ ~3 h after the start of perfusion. To increase gene transfer efficiency (7, 16), the hearts were perfused with medium containing histamine and serotonin (10 μmol/l each) that was drained off after passage through the heart. After 10 min, the perfusion was stopped and the virus was injected directly into the tube above the heart. Perfusion was started for 1 min, stopped for another 2 min, and periods of continuous and halted perfusion were repeated twice as described by others (7, 16). The medium containing the virus was collected in a 50-ml reservoir and recirculated until the end.

Inotropic response to isoprenaline. In the series of experiments designed to evaluate the influence of a 24-h perfusion on force of contraction and inotropic response to β-adrenergic agonists, isoprenaline was infused for 15 min 3 h after the start of perfusion, the perfusate was drained, isoprenaline was washed out for 20 min, and recirculation was reestablished. This procedure was repeated after 21 h of perfusion. In the series of experiments designed to test the influence of virus infection on β-adrenergic responses, isoprenaline was applied only 21 h after infection. To enlarge the inotropic response, the perfusion medium in this series of experiments was exchanged to a low-calcium (1 mmol/l) Tyrode solution before application of isoprenaline. This reduced force of contraction by ~80% (see Fig. 6) but yielded enhanced inotropic response to isoprenaline.

Histological procedures. Hearts were fixed in 4% formaldehyde in PBS overnight at 4°C, either directly after excision or after the 24-h perfusion, and subjected to standard paraffin embedding. Slices (4 μm) were cut and stained with hematoxylin and eosin (H&E) according to standard protocols. To determine transfection efficiency after adenoviral gene transfer with Ad5LacZ, hearts were not fixed but immediately frozen in liquid nitrogen and embedded in TissueTek, and 10-μm slices were cut. Sections were washed with PBS, fixed with 70% ethanol for 1 min, washed with PBS twice, and incubated with LacZ assay solution [20 mmol/l K₂[Fe(CN)₆], 20 mmol/l K₃[Fe(CN)₆], 2 mmol/l MgCl₂ 6H₂O, 0.02% Igepal CA-650, 0.01% sodium deoxycholate, and 1 mg/ml X-Gal] for 10 min. Slices were rinsed with PBS, postfixed in 4% formaldehyde in PBS, and counterstained with H&E.

Statistical analysis. Data were calculated as arithmetic means ± SE and analyzed using an unpaired t-test. A P value of <0.05 was considered significant. n indicates the number of independent experiments.

RESULTS
Characterization of the 24-h Langendorff perfusion model sterility. An inherent problem of 24-h perfusions of biological specimens excised under semisterile conditions in a half-open system is bacterial and fungal infection. Indeed, initial experiments with medium containing only Pen-Strep at standard concentrations showed severe contamination in both the medium and the heart. Inclusion of four sterile one-way filters (Fig. 1), exclusive use of silicone tubing and glass connectors, autoclaving the entire unit once a week, i.e., for perfusion of eight hearts, and inclusion of amphotericin B at one-third of the standard concentration eliminated bacterial or fungal growth (10 samples taken during several weeks, data not shown).

Fig. 2. Functional performance of Langendorff-perfused rat hearts (n = 23) over 24 h. A: spontaneous and isoprenaline-stimulated beating rate 3 h and 24 h after the start of perfusion. bpm, Beats/min. B: spontaneous and isoprenaline-stimulated twitch tension (TT) 3 h and 24 h after the start of perfusion. C: perfusion pressure over the entire 24-h period. *P < 0.05 vs. 3 h.
Online measurement of beating rate, force of contraction, and perfusion pressure. Force of contraction was measured by connecting a hook in the apex of the perfused heart with a self-made force transducer (Fig. 1). Online recording was interrupted during the night by applying a weight of 4 mN to the apex (removed the next morning) because it was shown to increase the reproducibility of force measurements after 24 h ($n=H110054$, data not shown). Under these conditions ($n=H1102123$), the hearts beat at a mean frequency of 145 beats/min 3 h after the start of perfusion and 137 beats/min 24 h after the start of perfusion (Fig. 2A). Isoprenaline (0.1 $\mu$mol/l) increased beating rate to 179 beats/min 3 h after the start of perfusion and 168 beats/min 24 h after the start of perfusion (Fig. 2A). Interestingly, force of contraction increased between 3 h and 24 h after the start of perfusion from 1.8 to 2.9 mN (+61%; Fig. 2B). Isoprenaline (0.1 $\mu$mol/l) increased force of contraction to 3.7 mN (+106%) 3 h after the start of perfusion and to 4.7 mN (+62%) 24 h after start of perfusion (Fig. 2B). In absolute terms, the increase in force of contraction was similar (1 mN). The perfusion pressure was measured online for 24 h and, after a short equilibration period with slightly higher pressures, remained constant at 1 ml/min (Fig. 2C). Perfusion at 2 ml/min raised pressures to 50 mmHg (declining over time to 30 mmHg) but did not lead to better contractile function after 24 h ($n=H110057–8$ per group; not shown). Because sterile filters obstructed, we found the 1 ml/min rate more suitable.

Conditions of perfusion. The medium was designed according to standard cell culture techniques, published protocols (14), and our experience. Not all of the supplements were systematically tested. First, amphotericin B at standard concentration reduced force of Fig. 3. Effect of medium composition on contractile function. Spontaneous and isoprenaline-stimulated TT 24 h after start of perfusion in the presence of 0.1 nmol/l ($n=H110057$ hearts) or 1.0 nmol/l ($n=H1100510$ hearts) triiodothyronine (T$_3$).

Fig. 4. Tissue properties of 24-h Langendorff perfusion of neonatal rat hearts. A: dry weight-to-wet weight ratio of native and 24-h perfused hearts. B: protein concentration in percentage of wet weight in native and 24-h perfused hearts. $n$, Number of hearts studied. C: photomicrograph of an hematoxylin and eosin-stained paraffin section of a native neonatal rat heart (day 2). D: 24-h perfused heart of same age; bar = 50 $\mu$m. E: representative Western blot and statistical analysis of actin, GAPDH, and calsequestrin (CSQ) in native and 24-h perfused neonatal rat hearts ($n=H110218$ each). $P<0.05$ vs. native.
contraction below 1 mN or hampered positive inotropic effect of isoprenaline (exclusion criteria) in five of seven experiments. Second, force of contraction and the positive inotropic effect of isoprenaline 24 h after the start of perfusion were 16% and 25% higher in hearts perfused with 1 nM triiodothyronine than with 0.1 nM (Fig. 3). Third, at a later stage of the experiments, a series of experiments showed that omitting insulin from the medium did not affect basal force of contraction but doubled the inotropic response to isoprenaline (n = 6 per group; not shown). Finally, addition of fetal calf serum or albumin was not compatible with the continuous bubbling of the medium.

Heart wet weight and dry weight, protein concentration, and histology. Given that interstitial edema represents a limiting factor of Langendorff-perfused adult hearts, several parameters were studied to quantify edema in 24-h perfused neonatal rat hearts. As shown in Fig. 4, the dry weight-to-wet weight ratio showed a 10% decrease compared with native, unperfused hearts from animals of similar age (Fig. 4A). Protein concentration decreased from 9.3 to 8.4% (~10%; Fig. 4B). Standard H&E histology showed a less compact tissue structure in the 24-h perfused hearts, an increase in interstitial space, and a decrease in myocyte diameter and eosinophilic staining (Fig. 4, C and D). However, both actin and calsequestrin, two markers of cardiac myocytes, remained unchanged when assayed as such or normalized to GAPDH (Fig. 4E). This indicates that the changes, if present, are minor.

Adenoviral gene transfer into 24-h Langendorff-perfused neonatal rat heart. Hearts were perfused with Ad5LacZ in a volume of about 5 ml under three times 2-min stop-flow conditions in the presence of histamine and serotonin. The virus was then recirculated for the rest of the time in a 50-ml volume. These conditions were chosen after initial experiments with different application modes and increasing concentrations of virus and were found to yield a high transfection efficiency throughout the hearts partly excluding the atria (Fig. 5). Importantly, the transfection efficiency of ~80% was stable in seven consecutive experiments. Histologically, β-galactosidase staining showed nuclear concentration, but the myoplasma was stained blue as well (Fig. 6). Virus infection did not affect beating rate (Fig. 7A) or force of contraction under basal or isoprenaline-stimulated conditions compared with uninfected controls studied in parallel (Fig. 7B).

DISCUSSION

We developed a new experimental model, the 24-h Langendorff-perfused neonatal rat heart, and showed that it is stable for >24 h and allows highly efficient
adenoviral gene transfer at relatively low virus concentration without apparent adverse effect on contractile behavior. It is relatively simple, inexpensive (complete setup ~11,000 Euro (circa $10,330), each run ~22 Euro (circa $21)), and robust, yet relatively complex and physiological and may therefore be suitable as a screening model for studying gene-function relationships ex vivo.

During the establishing phase we found several parameters to be critical. First, air bubbles were avoided by scrupulous control of the tubing. In addition, bubble traps directly above the heart are essential. Second, continuous filtering of the perfusate is necessary when using a recirculating mode and can easily be achieved with one-way filters. Third, sterility is difficult to ensure because the heart is manipulated and is beating in an open reservoir. For logistical reasons we did not perform manipulations under the sterile hood. Thus germs were present from the beginning of the experiment and likely entered the perfusate continuously during the 24 h in a normal cell culture laboratory. We solved this problem by autoclaving the entire perfusion system once a week and by inclusion of Pen-Strep and amphotericin B. The latter was used at one-third of the recommended concentration for cell culture to avoid toxicity. In addition, sterile filters in the circulation were changed for each experiment. Fourth, medium composition is certainly critical, but we did not extensively test different compositions. Instead, we modified an existing protocol (14) according to our cell culture experience. Two statements can be made: increasing triiodothyronine to 1 nmol/l improved contractile preservation, and interestingly, omitting insulin improved the inotropic response to isoprenaline (note that all experiments depicted here have been performed in the presence of insulin). Initial experiments with fetal calf serum did not indicate improvement and added the problem of foaming in the continuously gassed reser-

Fig. 6. Histological analysis of Ad5LacZ-infected 24-h Langendorff-perfused neonatal rat hearts. Photomicrograph of a paraffin section (4 μm) of a β-galactosidase-stained, formaldehyde-fixed (4%, overnight), PBS-rinsed (overnight) heart. Section was counterstained with hematoxylin and eosin. Note that with this technique, histological preservation was superior, but transfection efficiency was underestimated. Bar = 30 μm.

Fig. 7. Effect of Ad5LacZ on contractile function of 24-h Langendorff-perfused neonatal rat hearts (n = 7). A: beating rate and contraction kinetics of hearts 24 h after the start of perfusion in the absence or presence of Ad5LacZ. dF/dt+ maximal increase in force over time (mN/s); dF/dt− maximal decrease in force over time (mN/s); T1, time from 10% to peak force development; T2, time to 90% relaxation. B: TT and response to isoprenaline 24 h after the start of perfusion in the absence or presence of Ad5LacZ. Force of contraction was determined in normal perfusion medium (medium), 45 min after change of perfusion to low Ca²⁺ Tyrode solution (tyrode), and 15 min after application of isoprenaline (0.1 μmol/l).
voir. Fifth, an important parameter of success is mechanical loading of the heart. We found the hearts to perform better when a constant weight was applied to the apex during most of the perfusion time and if the heart can perform work. Even though we did not systematically study contraction mechanics, it appears obvious that the heart performs “auxotonic” work when lifting a weight with each heart beat. This is more physiological than either an isometric or isotonic mode of contraction. The weight of 4 mN (400 mg) is about 10 times the weight of a neonatal rat heart. Finally, the conditions of virus infection were chosen according to recent studies in adult heart models (8, 18) showing improvement of transfection efficiency with vasodilating receptor agonists such as histamine, serotonin, bradykinin, and vascular endothelial growth factor, as well as by stop flow. We did not systematically study the impact of these parameters on transfection efficiency in our model. One may argue that the stop-flow method induced preconditioning. However, the similar values for force of contraction in the virus-perfused (stop flow) and nonperfused (continued flow; compare Figs. 2 and 3 with 7B) and the fact that 21 h separated stop flow from force measurements argue against a significant impact of this intervention.

Even though most of the parameters studied demonstrated good performance of the model, limitations exist. An obvious, inherent problem of the model is that the neonatal rat heart is immature, e.g., lacks a well-developed sarcoplasmic reticulum/T-tubular system (2). Thus it may not be optimal for studying the impact of sarcoplasmic reticulum-related genes. Moreover, a relatively low heart rate and short action potential differ significantly from the mature rat heart. Secondly, 24 h of perfusion induced edema, thinning of myocytes, and a reduction in heart weight. This indicates some damage. Yet, with about 10% this effect was relatively modest and apparently did not affect perfusion pressure, contractile function, or beating rate. Actin and calsequestrin concentration also remained constant, arguing against significant myofilament degradation. Accordingly, force of contraction significantly increased. We believe that the latter does not indicate real improvement of contractile force but rather some geometric adaptation to the direction of strain on the heart. This relates to another principal shortcoming of the model, namely that contractile function is not measured by a balloon but by a hook in the apex and a force transducer. The latter was constructed not to measure force of contraction in an isometric manner, but rather auxotonic. Thus it behaves like a spring and allows the heart to work (see above). We are aware that this setup is not optimal in terms of muscle physiology, but installment of a balloon in the left ventricular cavity of a neonatal rat heart, one-third of the size of a mouse heart, is technically demanding and not suitable for screening purposes (at present 4 hearts are run in parallel). Given the well-developed inotropic response to isoprenaline (Fig. 6B) we feel that the present setup is a good alternative.

When compared with existing experimental ex vivo systems, the 24-h perfused neonatal rat heart has certain advantages and disadvantages that should be taken into consideration when designing an experiment. Whereas it is clearly more laborious than culturing and infecting neonatal or adult cardiac myocytes, it undoubtedly allows better functional evaluation of contractile parameters. This new method is easier to perform and control (in a reproducible manner) than the model of direct virus injections in the living heart or the cultured trabecular system in which sufficient transfection efficiencies are only obtained with >100 times more virus (17). We feel that particularly the ease of virus infection, the reproducibility of high transfection rates at low titers, and the absence of signs of virus toxicity are important advantages. With one virus preparation from 40 dishes (145 mm) of HEK cells, we routinely get 1 × 10^{11} bavs. Thus ~100 hearts can be studied with one virus preparation. The latter can be thoroughly titered, which is critical for quantitative statements and comparison of different experiments. Another advantage is the efficiency in terms of animal usage. Existing cell isolation methods yield about 2 × 10^6 cells/neonatal rat heart, the number of cells necessary to plate one 60-mm cell culture dish at high density. A principal advantage of the intact heart is that cardiac myocyte function can be studied in the intact three-dimensional context, devoid of the confounding factors of cell isolation. Finally, it integrates the function of the entire cardiac cell population, whereas isolated cells necessarily represent a selection of cells that survive the harsh conditions of enzymatic cell isolation.

In conclusion, the new model should allow studies on the function of genes in a cardiac context and the impact of quantitative changes in gene expression. As such, it may be a useful complementation of existing experimental models.

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DISCLOSURES
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