Effect of increased expression of cytoskeletal protein vinculin on ischemia-reperfusion injury in ventricular myocytes

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Wei, Hongguang, Thomas L’Ecuuyer, and Richard S. Vander Heide. Effect of increased expression of cytoskeletal protein vinculin on ischemia-reperfusion injury in ventricular myocytes. Am J Physiol Heart Circ Physiol 284: H911–H918, 2003; 10.1152/ajpheart.00525.2002.—The transition from reversible to irreversible ischemic injury (ischemia-reperfusion, I/R) occurs coincident with the loss of vinculin, a cytoskeletal protein involved in the attachment of the myofibrils to the sarcolemmal membrane. If the loss of vinculin were critical to the development of I/R, then increased levels of vinculin would be predicted to delay the onset of irreversible injury assuming that the protein is functional and localized to the proper subcellular site. The present study determined whether increased expression of vinculin, specifically in the cytoskeletal compartment, would provide protection from I/R injury. Neonatal rat myocytes were cultured and infected with a newly created replication-deficient adenovirus driving the expression of vinculin. I/R was induced with chemical inhibitors of glycolysis and mitochondrial respiration. Irreversible cell injury was assessed with lactate dehydrogenase (LDH) release. Virus-infected myocytes expressed significantly more vinculin in the cytoskeletal fraction and increased the expression of paxillin but sustained the same amount of injury in response to simulated I/R as control cells (n = 4; P = not significant, paired t-test). Hypothermic I/R (ischemia at 25°C) resulted in a significant reduction in LDH release (P ≤ 0.02; n = 4). Virus-mediated overexpression of vinculin does not appear to represent a rational approach to overcoming I/R injury.

cytoskeleton; paxillin; myocyte; adenovirus

SEVERE MYOCARDIAL ISCHEMIA results in irreversible injury to myocytes and is manifest classically as coagulation necrosis. Despite many years of active research, the exact series of subcellular events underlying the transition from reversible to irreversible injury remains elusive. It is known that certain interventions are capable of modulating or delaying the onset of irreversible injury in experimental model systems such as hypothermia (1, 11), calcium channel blockade (13, 16, 26), and, more recently, ischemic preconditioning (12, 15, 18, 21). However, even in experimental model systems, the mechanisms responsible for protection are not fully known.

Previous studies showed that cytoskeletal proteins may play an important role in the transition from reversible to irreversible injury (6, 9, 20, 25). For example, the loss of immunofluorescent staining for vinculin correlates temporally with the onset of myocardial fragility in isolated, perfused rat hearts (6, 25) and in vitro ischemic slices of canine myocardium (20). These studies led to the “cytoskeletal hypothesis of injury,” which states that the transition from reversible to irreversible myocyte injury is caused by the development of critical lesions in the myocyte cytoskeleton and that these lesions are exposed by the reintroduction of normal oxygen and arterial blood flow (i.e., reperfusion), which causes massive cell swelling as well as resumption of oxidative metabolism and cellular contraction.

Cytoskeletal proteins are important in the myocardium because they are involved in the anchoring of the sarcolemmal membrane to the underlying contractile apparatus. When these support structures are compromised, the overall fragility of the muscle cell (myocyte) is increased. The correlation of increased fragility with loss of cytoskeletal support is seen best in muscular dystrophy (4). In Duchenne muscular dystrophy, the loss of dystrophin, a cytoskeletal protein, is the consequence of an inherited genetic defect. Clinically, this defect is manifest primarily as a progressive loss of skeletal muscle fibers. However, dystrophin is also present in cardiac myocytes. Because dystrophin serves as a mechanical link between the myocyte cytoskeleton, through the dystrophin-associated glycoprotein complex, and the extracellular matrix, injury and cell death can occur when this link is disrupted by repetitive contraction (5). Therefore, Duchenne muscular dystrophy as well as other forms of hereditary muscular dystrophies can exhibit a significant cardiac phenotype, indicating that loss of cytoskeletal proteins can have important functional consequences in cardiac muscle.

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However, to date most of the support for the cytoskeletal hypothesis in experimental cardiac injury has come from indirect data, which have demonstrated a correlation of the loss of specific cytoskeletal proteins and/or immunofluorescent staining for cytoskeletal proteins with the onset of irreversible injury. The converse experiment, i.e., increasing expression of cytoskeletal proteins in an attempt to protect cells from ischemic injury, has not been possible. With the development of replication-deficient adenoviral techniques, it is now possible to transiently increase the expression level of a variety of proteins including cytoskeletal proteins and therefore test directly whether targeted increases in the expression of cytoskeletal proteins result in protection from irreversible cell injury.

The purpose of the present study was twofold: first, to increase the expression level of a cytoskeletal protein previously shown to be important in irreversible myocyte injury, i.e., vinculin; and second, to determine whether increased expression of vinculin would provide protection from simulated ischemia-reperfusion (I/R) injury.

METHODS

All experiments reported here conformed to the standards in the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Pub. No. 85-23, Revised 1985).

Isolation of neonatal myocytes. For each isolate, the ventricular portions of six or seven hearts from 1- to 2-day-old rats were pooled and gently agitated overnight at 4°C with trypsin (0.1 g in 50 ml) in HBSS. The next day, the myocytes were digested further with serial incubations in collagenase (0.1 g in HBSS). The final cell isolate was centrifuged for 3 min at 1,000 rpm and 4°C. The resulting supernatant was transferred to a 50-ml conical tube, and centrifuged again for 4 min at 1,000 rpm and 4°C. The resulting supernatant was discarded, and the cells were resuspended in ice-cold DMEM, containing DMEM supplemented with 10% FBS and antibiotics (600 U/ml penicillin-streptozotocin and 30 μg/ml gentamicin) and cultured in standard six-well plates (Corning, Corning, NY). Approximately 3.0 × 10^6 cells were placed in each well, and cells were allowed to attach for 1 h to reduce the number of fibroblasts in the final preparation. After 1 h, the cells were removed and transferred to a fresh plate with fresh medium before initiation of the experimental protocol. Immunofluorescent staining for muscle-specific actin confirmed that >95% of the plated cells were myocytes.

Construction of recombinant adenovirus. Full-length mouse vinculin cDNA (a generous gift from Dr. Eileen D. Adamson, Burnham Institute, La Jolla Research Center, La Jolla, CA) was inserted into the E1 region of an adenoviral vector (pADvantage) The pADvantage vector and the shuttle vector were a gift from David W. Souza of Genzyme (Framingham, MA). The cloning procedure used is described in detail in the original reference by Souza and Armentano (19). An empty adenovirus was constructed in parallel as a control for the effects of adenoviral infection in the target cells.

Briefly, chicken vinculin cDNA was cloned into the multiple cloning site of the shuttle plasmid pSV2-ICEU1. The shuttle plasmid was constructed within a pBluescriptSK (+) backbone and contained the cytomegalovirus promoter (which contains a mutation that eliminates the Sp6BI site), multiple cloning sites, and the bovine growth hormone poly(A) signal. The ICEU1 fragment from the shuttle plasmid was cloned into the E1 region of the pADvantage plasmid that includes the adenovirus serotype 2 genome and a polylinker containing unique restriction sites that supplanted the Ad2 E1 region. The resulting plasmid was digested with SnaBI and was transfected into the human embryonic kidney cell line 293 (HEK293 cells) with the LipofectAMINE PLUS reagent package (GIBCO, Grand Island, NY). The resulting plaques were picked 7-10 days after transfection with 10-mm cloning cylinders. Viral stocks were generated by infection of HEK293 cells, harvest of the cells, and purification by CsCl ultracentrifugation. The titer of viral stocks was determined by either optical measurement or plaque assay.

Experimental design and protocol. Myocytes were divided into three groups: control, experimental virus infected, and control virus infected. Three subcellular compartments were isolated with replication-deficient adenovirus containing the cDNA of chicken vinculin at a multiplicity of infection (MOI) of 1 (see Construction of recombinant adenovirus). Another group of myocytes from the same isolation was infected with “empty virus,” a similar strain of replication-deficient adenovirus that did not contain the vinculin coding sequence (also at an MOI of 1). Finally, one group of myocytes was cultured without any adenovirus infection. After 48 h of incubation, the three groups of myocytes were each split into two equal halves (i.e., 3 wells from each 6-well plate). One-half of each plate was used for Western blot analysis of vinculin protein expression, and the other half was used to test for cardioprotection from simulated I/R injury. To induce ischemia, the culture medium was removed and replaced with fresh medium containing 3.0 mM iodoacetic acid (IAA) to inhibit glycolysis and 3.0 mM amobarbital (amytal) to inhibit mitochondrial respiration. After 240 min of incubation, the cells were assayed for cell death (see below) or the ischemic buffer was exchanged with fresh hypotonic oxygenated culture medium without the chemical inhibitors for 30 min to simulate reperfusion. Previous studies showed that hypotonic reperfusion best simulates the effects of reperfusion in ischemic tissue (7, 25). The resulting proportion of live and dead cells in both groups was determined by measuring lactate dehydrogenase (LDH) release with a commercially available kit (Sigma, St. Louis, MO).

To confirm that cellular protection could be measured with the described system (i.e., cultured neonatal rat myocytes subjected to simulated I/R), a separate set of control experiments were conducted. In these experiments, noninfected myocytes were cultured for 48 h before exposure to simulated I/R. Simulated ischemia was conducted at two temperatures, 37°C (control) and 25°C (hypothermia), to determine whether hypothermia protected against lethal cell injury. Reperfusion was carried out at 37°C in both groups.

In some experiments, cells were infected in an identical fashion and used for qualitative studies. These studies used Western blots to assess the subcellular distribution of vinculin. For these studies, cells were processed with a protocol designed to render three subcellular compartments (2): cytosolic, membrane, and cytoskeletal (see Cell fractionation procedures for details).

Cell fractionation procedures. Cytosolic, membrane, and cytoskeletal fractions were prepared by a modification of the method described by Goldberg et al. (8). Briefly, ventricular...
myocyte cultures were washed with PBS and then immediately lysed in 25 μl of a protease inhibitor cocktail (Sigma) and 0.2 ml of ice-cold homogenization buffer [final concentration (in mM) 20 Tris-HCl (pH 7.5), 2 EDTA, 2 EGTA, 3 β-mercaptoethanol, 0.1 sodium vanadate, and 50 NaF]. The isolate was passed through a 26-gauge needle several times to enhance cell lysis and then centrifuged at 15,000 g for 10 min. The supernatant was removed (soluble fraction), and the pellet was resuspended in 0.1 ml of homogenization buffer containing 1% Triton X-100 to solubilize membrane proteins. After shaking on ice for 30 min, the Triton X-100-insoluble fraction was removed and centrifuged at 15,000 g for 10 min. The resulting pellet was solubilized with 0.8% SDS to obtain a cytoskeletal fraction.

Western blot procedures. Three wells of each six-well plate (the wells not subjected to simulated ischemia) were harvested for protein analysis with standard Western blot techniques. Briefly, cells were washed twice with PBS, lysed with 0.6% SDS, and stored at −20°C until analysis. Samples were thawed, and each lane was loaded with an equal amount of protein [as determined by bicinchoninic acid (BCA) assay; Pierce] and subjected to SDS-protein electrophoresis with 7.5% acrylamide gels. After electrophoresis, the proteins were transferred to nitrocellulose membranes and the membranes were blocked with 5% nonfat milk in Tween 20-Tris-base sodium for 2 h at room temperature and then incubated overnight at 4°C with a monoclonal anti-human primary antibody to vinculin (Sigma) or a monoclonal anti-chick paxillin antibody (Transduction Laboratories) at a dilution of 1:5,000. The next day, the membranes were incubated with an anti-mouse IgG (Roche Diagnostics) at a 1:10,000 dilution. Vinculin and paxillin protein expression was detected with a chemiluminescence detection system (Amersham, Arlington, IL). The protein concentration of each sample for Western blot was determined by use of a BCA protein assay kit. Equal amounts of each protein to be compared were loaded to ensure appropriate comparison of protein expression/content level.

Immunoprecipitation. For these experiments, myocytes were cultured on standard 100-mm culture plates. After infection with virus, cells were rinsed with PBS at room temperature followed by the addition of 0.6 ml of RIPA buffer (1% PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS) containing protease inhibitors (10 μl/ml PMSF and 2 μg/ml aprotinin). The myocytes were scraped from the culture dish, passed through a 26.5-gauge needle, and placed in a microcentrifuge tube. This procedure was repeated again to ensure total extraction of all cells. The lysates were incubated on ice for 30 min and centrifuged at 10,000 g for 10 min at 4°C. After the whole cell lysate was preceeded, ~1 ml of the total cell lysate (containing equal amounts of protein) was incubated with 5 μl of goat polyclonal anti-vinculin antibody for 1 h at 4°C. Twenty microliters of protein A/G agarose were then added, and the lysate was rocked gently overnight at 4°C. The cell pellet was collected by centrifugation at 1,000 g for 5 min at 4°C and washed four times with RIPA buffer. After the final wash, the supernatant was discarded and the pellet was resuspended in sample buffer and subjected to routine 7.5% SDS-PAGE. The separated proteins were transferred to nitrocellulose membranes and probed for paxillin with a mouse monoclonal antibody (1:5,000 dilution; Transduction Laboratories) followed by an anti-mouse (IgG) secondary antibody conjugated to peroxidase (1:1,000 dilution; Roche Diagnostics). Membranes were probed with the chemiluminescence system described in Western blot procedures.

Immunofluorescence studies. In some experiments, cells were infected in an identical fashion and used for qualitative studies. These studies used immunofluorescent techniques to assess adenoviral infection efficiency. Briefly, neonatal cardiomyocytes were plated on chamber slides instead of six-well plates. After 48 h of infection with virus, cells were fixed with 3% paraformaldehyde in PBS and permeabilized with 0.5% Triton X-100. The fixed cells were sheared off the substrate with a jet of PBS to expose the ventral cell membrane for better visualization of the vinculin organization. The primary antibody was a mouse monoclonal anti-human antibody to vinculin (Sigma). The secondary antibody was a fluorescein-labeled goat anti-mouse IgG antibody. The cells were examined by epifluorescence with a Nikon TE300 inverted microscope.

LDH assay. To determine the amount of cell injury induced by the simulated I/R protocol, release of LDH was measured. LDH is normally retained in the cytosol until the sarcolemmal membrane is ruptured, after which it is free to diffuse into the surrounding media. After completion of the I/R protocol, the ischemic buffer in each well of the culture dish was replaced for LDH. The attached cells in each well were extracted, and the resulting extracts were analyzed for LDH. Total LDH was considered as the sum of the LDH released into the media during the I/R protocol plus the residual LDH present in the attached cells. The percent LDH release was calculated by dividing the amount released into the media by the total LDH (released plus cellular content) for each experimental group. This allowed the amount of LDH release to be normalized for the number of cells present in each dish. Each well was assayed in duplicate, and the results were averaged into one value for each group.

Statistics. Each myocyte isolate generated control and experimental groups, and therefore each isolate served as its own control. All data are expressed as means ± SE. Statistically significant differences between groups were tested with a paired t-test analysis. In all analyses, a P value <0.05 was considered statistically significant.

RESULTS

Isolation of neonatal rat ventricular myocytes. Testing the hypothesis underlying the present study required a selective increase in vinculin expression in ventricular myocytes without compromise of viability. With techniques previously reported (23, 24), we were routinely able to isolate large numbers of viable neonatal rat ventricular myocytes. After establishing an effective isolation procedure (as described in METHODS), we sought to determine whether cultured neonatal rat ventricular myocytes could be directed to increase a transgenic protein with replication-deficient adenoviral techniques.

Subcellular distribution of vinculin protein expression in neonatal rat myocytes. Infection of neonatal and adult rat myocytes with replication-deficient adenoviral constructs results in increased expression of transgenic proteins (14, 23). However, it is not clear where in the cell the increased protein expression occurs. Therefore, one of the purposes of this study was to determine the subcellular distribution of increased vinculin expression in neonatal rat ventricular myocytes. The optimal infection with virus was determined in preliminary experiments. As shown in Fig. 1, infection with the vinculin adenovirus (vin) resulted in a large
increase in myocyte vinculin content as assessed by immunofluorescence. Importantly, the viability of infected myocytes did not differ significantly from that of control cells over 3 days of postinfection culture (LDH data not shown).

Because vinculin is a cytoskeletal protein, we expected that most of the increased expression (measured after 48 h) would occur in the cytoskeletal fraction. Infected myocyte cultures were fractionated followed by Western blotting for vinculin. As shown in Fig. 2, myocytes infected with vin at 1 MOI (calculated from plaque-forming units of the virus and the number of cells plated per well) expressed vinculin in all three subcellular compartments (soluble or cytosolic, membrane, and cytoskeletal) in relatively equal proportions. Because the newly overexpressed vinculin did appear in the cytoskeletal compartment, it was concluded that the model system and the virus would provide a useful test of the underlying hypothesis. In addition, the results shown in Fig. 2 provide independent confirmation that the absolute level of vinculin expression was increased specifically by the vin-containing adenovirus (i.e., compared with empty virus).

After verification that vinculin was present in the cytoskeletal fraction of the myocytes, it was important to determine whether the newly expressed vinculin was interacting in a biologically meaningful way with normal vinculin-associated proteins. Paxillin is a multidomain focal adhesion protein that contains binding sites for many structural and regulatory molecules such as vinculin and focal adhesion kinase (22). By immunofluorescent microscopy, myocytes overexpressing vinculin also stained brighter for paxillin compared with control myocytes and myocytes infected with empty virus (data not shown). This was confirmed by quantification of Western blots (Fig. 3), which showed that cells overexpressing vinculin contained 25% more paxillin in the soluble fraction and 8% more paxillin in the membrane fraction compared with control myocytes. Together, these results show that virally induced vinculin is capable of interacting and/or binding with a usual binding partner (paxillin) and furthermore suggest at least that vinculin may have an important regulatory effect on paxillin expression.

**Effect of simulated I/R on subcellular vinculin content and distribution.** We examined the effect of simulated ischemia on the subcellular distribution of vinculin to determine whether ischemia induces a redistribution and/or loss of vinculin similar to that originally described in intact rat and dog myocardium (20, 25). Figure 4 compares the subcellular vinculin distribution from oxygenated control myocytes with that of myocytes subjected to 240 min of simulated ischemia. Compared with normoxic control myocytes, myocytes

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**Fig. 1.** Micrographs of cultured neonatal rat myocytes. A: control neonatal rat ventricular myocytes 48 h after initiation of culture. Control cells show baseline vinculin expression. B: ventricular myocytes infected with replication-deficient adenovirus designed to increase expression of vinculin. Note the marked increase in immunofluorescent staining compared with control cells (images shot at same exposure time).

**Fig. 2.** Myocytes were extracted and separated into 3 subcellular compartments as described in METHODS. Control myocytes (Con) were incubated under oxygenated conditions for 3 days. Two separate plates from the same isolate were infected with either a replication-deficient adenovirus designed to increase vinculin expression (Vin) or empty adenovirus as a control for viral infection. Infection with Vin adenovirus resulted in a large increase in vinculin expression in all 3 compartments compared with control myocytes. Myocytes infected with the empty adenovirus lacking the coding region failed to exhibit an increase in vinculin expression. Cyto, soluble or cytosolic fraction; Mem, membrane fraction; Csk, cytoskeletal fraction.
subjected to ischemia for 240 min showed a 23% decrease in cytoskeletal vinculin content. Membrane vinculin content decreased an average of 63% compared with control, whereas soluble vinculin content increased by 59%. A similar decrease in membrane vinculin content was observed in overexpressing cells in response to ischemia. However, because of the large increase in soluble vinculin expression in infected cells, we did not detect a significant increase in the soluble fraction of vinculin in response to ischemia (data not shown).

Effect of increased vinculin expression and hypothermia on response to simulated I/R injury. Having established that increased expression of vinculin occurs in the cytoskeletal fraction of cultured ventricular myocytes and that newly synthesized vinculin colocalizes with an important adapter protein (paxillin), we sought to determine whether increased vinculin expression would protect isolated myocytes against simulated I/R. Previous studies showed that incubation of myocytes with inhibitors of both glycolysis (IAA) and mitochondrial respiration (amytal) causes significant irreversible injury (23, 24). Myocytes were infected with vinculin-expressing or empty adenovirus for 48 h and then subjected to I/R. After 240 min of simulated ischemia with IAA plus amytal, LDH release was measured (nonreperfused group) or buffer containing IAA and amytal was exchanged with hypotonic medium (control drug-free medium diluted 1:1 with sterile water; reperfused group) for 15 min. After 240 min of ischemia, there was no difference between any of the three groups [control 31.1 ± 4.7%, vinculin 36.1 ± 6.9%, and empty virus 36.2 ± 6.4% LDH release, respectively (n = 4); P = not significant; Fig. 5]. The addition of 30 min of hypotonic reperfusion after ischemia significantly increased LDH release in all three groups. LDH release in control myocytes increased from 31.1 ± 4.7% to 86.0 ± 4.3% (P < 0.001 vs. 240-min ischemia alone; Fig. 5). Similarly, LDH release in vinculin-infected and empty virus-infected myocytes increased to 90.5 ± 4.5% and 89.2 ± 4.4%, respectively (both P < 0.001 compared with corresponding ischemia-only groups; Fig. 5). Thus increased vinculin expression failed to protect from simulated I/R.

Effect of hypothermia on I/R injury. Because increased vinculin expression unexpectedly failed to protect from I/R injury, we wanted to ensure that protection could be measured in our model system in the absence of adenoviral infection. Accordingly, myocytes were subjected to the same I/R protocol but the 240-min incubation was carried out at 25°C instead of 37°C (hypothermia). Myocytes subjected to I/R at 25°C sustained significantly less cell injury compared with myocytes subjected to I/R at 37°C [64.2% vs. 48.6% (n = 4); P < 0.05; Fig. 5]. This result indicates that cardioprotection can be measured in this model system.

Fig. 3. To assess whether transgenic vinculin retains normal biological activity, the expression of paxillin was determined in control (C), vinculin-overexpressing, and empty virus-infected myocytes. Twenty-four hours after isolation, myocytes from the same isolate were infected with Vin virus or empty virus and then maintained under control conditions for an additional 2 days (3 days total in culture). A separate plate of noninfected myocytes from the same isolate was cultured for 3 days as a control group. After 3 days, myocytes were extracted and separated into 2 compartments, cytosol (soluble) and membrane, and then probed for paxillin. Equal amounts of protein were loaded in each lane to allow for direct comparison of the amount of paxillin present. Compared with control myocytes and myocytes infected with empty virus, Vin-infected myocytes contain more paxillin, indicating that myocytes overexpressing vinculin bind more paxillin and/or cause upregulation of paxillin expression.

Fig. 4. Comparison of vinculin expression in control (normoxic, –) myocytes and myocytes subjected to ischemia (+) for 240 min (cells from same isolate). After ischemia, cells were extracted and separated into 3 compartments (soluble/cytosol, membrane, and cytoskeletal) as described in METHODS. Myocytes subjected to ischemia showed a reduction in detectable vinculin in both the membrane and cytoskeletal compartments. The soluble fraction showed an increase in detectable vinculin and an increased number of smaller immunoreactive bands. Western blot shown is representative of 2 separate experiments (n = 2).

Fig. 5. Myocytes were subjected to ischemia alone (I) or ischemia-reperfusion (I/R) as outlined in METHODS. All cells were exposed to hypotonic reperfusion where reperfusion is indicated. y-Axis indicates the percentage of total lactate dehydrogenase (LDH) release. Expression of vinculin did not provide protection compared with control myocytes after 240 min of ischemia alone or 240 min of ischemia followed by reperfusion. Hypotonic cell swelling along (i.e., “reperfusion” of nonischemic myocytes) did not cause injury. Hypothermic ischemia (I/R carried out at 25 vs. 37°C; right) provided significant protection, indicating that the model system is capable of detecting a cardioprotective effect. NS, not significantly different.

*Significant difference between control and hypothermic groups (P < 0.05); n = 4 for each data point.
DISCUSSION

The major findings of this study were 1) structural cytoskeletal proteins can be expressed in cultured myocytes, localize in the targeted (i.e., cytoskeletal) subcellular compartment, and associate with normal partner proteins; 2) the simulated I/R model system used in these studies shows increased injury in response to reoxygenation and/or reperfusion and is capable of detecting cardioprotection; and 3) increased expression of vinculin fails to protect the myocytes from simulated I/R injury. In addition, the study provides intriguing preliminary data that vinculin expression may somehow regulate paxillin expression in myocytes.

Role of cytoskeletal proteins in irreversible ischemic injury. It has been known for many years that cytoskeletal proteins play an important role in lethal myocardial ischemic injury (6, 10, 20, 23). Early studies showed that the loss of immunofluorescent staining for vinculin, a costameric cytoskeletal protein, correlated with the onset of irreversible cell injury in both adult dog and rat myocardium. These results led to the hypothesis that the critical lesion in irreversible myocyte injury is the development of lesions in the cytoskeleton that in turn lead to increased cell fragility. If this hypothesis is correct, then it would be predicted that either decreasing the total amount or the rate of vinculin loss or, contrarily, increasing the myocyte concentration of vinculin should reduce or retard irreversible cell injury. The underlying hypothesis of the present study was that increasing myocyte vinculin levels would result in a reduction in lethal cell injury. To test this hypothesis, we developed a new adenoviral construct designed to increase the expression of vinculin. After confirming the adenovirus-increased expression of vinculin, we tested for the ability of the increased vinculin expression to provide cardioprotection with a model of simulated I/R in cultured neonatal rat myocytes.

Subcellular localization of vinculin in myocytes. Few studies to date have determined the subcellular localization of transgenic protein expression in myocytes. To determine subcellular vinculin localization, we used differential centrifugation to generate three subcellular compartments after extraction of the cells: cytosolic, membrane/nuclear, and cytoskeletal (2). The results showed that vinculin expression was increased in all three subcellular fractions including the cytoskeletal compartment (Fig. 2).

Biological function of vinculin. In addition to targeting the protein of interest to the correct subcellular location, it is important to attempt to determine whether or not the protein associates normally with other proteins and/or functions the same as the native protein. In the case of a structural protein such as vinculin, it is more difficult to determine whether its “function” is normal. However, it is possible to examine whether it localizes and/or binds proteins normally associated with native vinculin. In this study, we examined the interaction of vinculin with paxillin. Paxillin is a multidomain adapter protein that has multiple functions in cell signaling (22). As shown in Fig. 3, myocytes expressing increased amounts of vinculin also expressed and/or contained more paxillin. Furthermore, immunoprecipitation of infected cells with vinculin brought down more paxillin in vinculin-infected cells than in control cells and cells infected with empty virus. These results provide important evidence that the vinculin expressed in virus-infected cells associated normally with paxillin, a protein known to bind native vinculin. Future studies with adenoviral techniques should examine the subcellular localization as well as the biological function of the expressed protein to ensure that the protein is being targeted to the correct subcellular site of action and is capable of assuming normal function once localized.

Effect of increased vinculin expression on response to simulated I/R. The goal of this study was to determine whether overexpression of vinculin would protect from simulated I/R injury. Providing a valid test of the hypothesis required development of a reliable model of I/R injury in cultured neonatal myocytes that reproduced as closely as possible features that have been described in intact hearts or animal models of I/R injury. Using metabolic inhibitors, we confirmed that simultaneous inhibition of both glycolysis and mitochondrial respiration results in significant lethal (irreversible) cell injury and that reperfusion exacerbates irreversible injury as evidenced by significantly larger amounts of LDH release (Fig. 5). More importantly, we confirmed that induction of ischemia resulted in a decrease in myocyte cytoskeletal vinculin, the vinculin thought to be most critical to overall myocyte stability. The vinculin level in the membrane fraction was also significantly reduced after ischemia. This is not surprising given the effect on cytoskeletal vinculin, because vinculin normally links the cytoskeleton to the sarcolemmal membrane. Compared with oxygenated myocytes, the amount of detectable vinculin in the cytosolic or soluble fraction increased in response to ischemia. Moreover, several smaller bands appeared in the soluble fraction after ischemia (Fig. 4). It is possible that these additional bands represent partial degradation products of vinculin. On the basis of the present results, we cannot be certain whether cytoskeletal vinculin was directly decreased by ischemia or whether ischemia induced redistribution from the cytoskeletal and/or membrane compartment to the soluble, cytosolic compartment. Nevertheless, the demonstration of ischemia-induced reduction and/or redistribution in vinculin levels, specifically in the cytoskeletal fraction, provides important verification of the model system because similar changes have been shown to occur in in vivo models of myocardial ischemia. Finally, we confirmed that hypothermia is effective in reducing ischemic cell injury, a well-known effect in many in vivo models of I/R injury (Fig. 5).

Despite the fact that hypothermia was capable of protecting myocytes from irreversible injury, as evidenced by a significant reduction in LDH release compared with control myocytes, overexpression of vincu-
lin did not alter LDH release and therefore did not protect the cells from simulated I/R injury.

Possible reasons for lack of cardioprotective effect. Possible explanations for the lack of vinculin-induced protection include that vinculin was not expressed in the proper subcellular compartment (i.e., the cytoskeletal compartment), that the vinculin that was expressed was not biologically active, or that in myocytes overexpressing vinculin the response to ischemia was altered such that vinculin was not altered and/or lost overexpressing vinculin the response to ischemia was similar to that described in other model systems (Fig. 4).

One definite possibility is that the model system used in these studies was not capable of detecting a significant cardioprotective effect with simulated I/R and LDH as an assay of lethal cell injury. However, this possibility seems unlikely because Fig. 5 shows that reducing the temperature of the ischemic insult caused a significant reduction in cell injury compared with myocytes rendered ischemic at 37°C. It is also unlikely that the use of adenovirus-directed overexpression by itself causes protection because the empty virus group showed no significant cardioprotection.

It is possible that neonatal myocytes may differ in important yet unrecognized ways from adult myocytes. The initial studies implicating vinculin in the development of irreversible injury were carried out in adult myocardium. There are well-known differences in the metabolism of neonatal and adult myocardium that may be important. Recent studies showed that some members of the heat shock protein family (i.e., HSP25/27) show protection in adult but not neonatal myocytes (14, 23). Therefore, it is possible that such effects could also apply to the overexpression of cytoskeletal proteins such as vinculin. It is also possible that other unknown cytoskeletal proteins that interact with vinculin need to be activated and/or increased in level to provide significant cardioprotection.

Future studies of cytoskeleton and irreversible myocyte injury. The fact that increased expression of vinculin did not protect cultured neonatal rat myocytes from simulated I/R injury does not diminish the importance of determining the role of the cytoskeleton in irreversible myocyte injury. Vinculin represents just one of many important cytoskeletal proteins that need to be examined. For example, there is emerging evidence that dystrophin may be important in cardioprotection (2, 5). Dystrophin represents a separate but equally important cytoskeletal protein that is critical in linking the sarcolemmal membrane to the underlying contractile apparatus (17), the most likely site at which a critical cytoskeletal lesion leading to increased myocyte fragility would occur. Future studies of the role of cytoskeletal proteins in irreversible ischemic injury should investigate dystrophin as well as other members of the myocyte cytoskeleton that link the contractile apparatus and the sarcolemmal membrane, such as utrophin and/or the dystroglycan-associated glycoprotein complex. Future studies should still consider whether increased expression of cytoskeletal proteins provides protection from irreversible injury but should also recognize the importance of targeting the protein to the correct subcellular compartment as well as whether the newly expressed protein retains its normal biological function.

Finally, it is possible that the reperfusion phase may provide important clues in deciphering the sequence of events in the transition from reversible to irreversible injury. For example, it is well known that in vivo I/R injury the osmotic stress of restoring normal, isomotic blood to the relatively hyperosmotic ischemic region is intimately associated with the initiation of sarcolemmal membrane rupture, release of intracellular enzymes, and myocyte death. Studies directed at modifying or manipulating the osmotic stress induced by reperfusion may yield important clues to the critical events underlying the transition from reversible to irreversible injury.

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