Adenovirus-mediated expression of p35 prevents hypoxia/reoxygenation injury by reducing reactive oxygen species and caspase activity

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Received 18 December 2001; accepted 25 March 2002

Abstract

Objective: This study aimed to examine the effects of adenovirus-mediated expression of p35, a baculovirus gene, on apoptosis induced by hypoxia/reoxygenation (H/R) in cardiomyocytes. Methods: Neonatal rat cardiomyocytes were infected with recombinant adenoviral vectors expressing p35 (Ad2/CMVp35) or no transgene (Ad2/CMVEV) and were then subjected to H/R. Separate groups of non-infected cardiomyocytes were treated with pharmacological caspase inhibitors or antioxidants. Cell viability, apoptosis, caspase activity, and cellular reactive oxygen species (ROS) were measured using various assays. Results: H/R decreased cell viability and increased cellular ROS levels, caspase activity, and cell apoptosis. Infection with Ad2/CMVp35 effectively inhibited the increase in cellular ROS levels, the activities of caspases 3 and 8, apoptosis, and cell death following H/R, whereas Ad2/CMVEV had no effect. Despite its ability to abolish the increase in caspase activity and partially inhibit apoptosis, the pan-caspase inhibitor ZV AD-fmk (100 μM) failed to significantly reduce cell death induced by H/R. N-acetyl-L-cysteine, an antioxidant, completely inhibited H/R-induced increase in cellular ROS levels, but reduced apoptosis and cell death by 30% only. Conclusions: Adenovirus-mediated expression of p35 effectively inhibits H/R-induced cardiomyocyte apoptosis by reducing cellular ROS levels and inhibiting caspase activity.

Keywords: Apoptosis; Gene therapy; Hypoxia/anoxia; Oxidative phosphorylation; Reperfusion

1. Introduction

A family of tightly regulated caspases is the central component of a specialized cellular machinery by which apoptosis is accomplished [1]. Activation of these proteases by proapoptotic stimuli results in disassembly of the cell. Increased cardiac cell death by apoptosis has been observed in animal models of ischemia/reperfusion [2]. Cultured cardiomyocytes also undergo apoptosis, which is accompanied by cytochrome c release from the mitochondria and caspase activation in response to components of ischemia/reperfusion, such as depletion of glucose and serum or simulated ischemia/reperfusion [3–5]. Pharmacological caspase inhibitors such as ZVAD-fmk have been shown to protect the myocardium against ischemia/reperfusion injury [6]. Although they effectively inhibit caspasess, their ability to inhibit disruption of the mitochondrial membrane potential and affect the ultimate fate of the treated cells remains controversial [7–11]. Such experimental observations highlight the role of mitochondria in cardiomyocyte cell death following ischemia/reperfusion, consistent with the notion that inactivation of caspasess and inhibition of the apoptotic morphology does not prevent cell death induced by some proapoptotic stimuli, including growth factor withdrawal, ultraviolet radiation, and Bax [12]. Although the precise initiating stimuli and signaling pathways for mitochondrial damage and subsequent commitment of the cells to death following
ischemia/reperfusion are not fully understood, one of the major mechanisms is provided by oxidative stress and the burst of reactive oxygen species (ROS) production [13,14]. ROS can directly cause DNA strand breaks with resultant targeting of these cells for p53-mediated apoptosis through upregulation of Bax [15,16]. ROS also induces the release of cytochrome c and precursors of caspases from the mitochondria into the cytoplasm [17–21]. Antioxidants and metallothioneins have been shown to decrease cardiac cell apoptosis following ischemia/reperfusion [22–25].

The baculoviral anti-apoptotic protein, p35, is a potent inhibitor of all the mammalian caspases except caspases 5 and 9 [26–28]. Expression of p35 prevents apoptosis induced by various stimuli in carcinoma cells, neurons, oligodendrocytes, and vascular smooth muscle cells [29–32]. Recent studies indicate that prolonged hypoxia-induced cell death, cytochrome c release, and caspase 3 activation are markedly reduced in cardiomyocytes isolated from transgenic mice expressing p35, compared to cardiomyocytes from wild-type mice [33]. In this study, we investigated the effect of adenoviral-mediated gene transfer of p35 on caspase activity, cellular ROS levels, apoptosis, and cell viability in neonatal rat ventricular myocytes following simulated ischemia/reperfusion. We demonstrated that adenovirus-mediated expression of p35 effectively protected cardiomyocytes against ischemia/reperfusion injury by reducing cellular ROS levels and by inhibiting caspase activity. The ability of p35 to reduce cellular ROS levels and intercept ROS-initiated signaling prior to or at the mitochondrial stage of suicidal death contributes to the protection of cardiomyocytes against simulated ischemia/reperfusion injury.

2. Methods

2.1. Construction of recombinant adenoviral vectors

The adenoviral vectors were constructed as described previously [32]. Briefly, Ad2/CMVp35 was an Ad2-based vector in which the E1 and E3 regions were deleted. Whereas the E1 region did not code any transgene, the p35 expression cassette driven by the human cytomegalovirus enhancer/promoter was inserted into the E3 region. The p35 gene was cloned by PCR from baculovirus genomic DNA. Ad2/EGFP encoding the green fluorescence protein (GFP) was also an Ad2-based vector in which the E1 region was replaced with a GFP expression cassette driven by the human cytomegalovirus enhancer/promoter. The E4 region except ORF6 was deleted. Ad2/CMVEV was constructed in a similar fashion as Ad2/EGFP, except that Ad2/CMVEV encodes no transgene.

2.2. Cell culture

This investigation conforms with principles outlined in the Declaration of Helsinki (Cardiovascular Research 1997;35:2–3). Neonatal rat ventricular cardiomyocytes were isolated and cultured as described previously [34]. Briefly, the cells were seeded at a density of $2.0 \times 10^5$ cells/cm² on rat collagen type I-coated dishes and maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% (v/v) fetal bovine serum for 48 to 72 h prior to use in the studies.

2.3. Detection of transgene expression

Cardiomyocytes were infected with Ad2/CMVp35, Ad2/EGFP or Ad2/CMVEV at various multiplicities of infection (MOI) for 6 h and maintained under normal culture conditions for an additional 42 h. GFP expression was assessed using light microscopy. For Western blot analysis, cells were lysed and protein was separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and then transferred to poly(vinylidene difluoride) membranes. The filters were blocked with 5% milk, followed by incubation with a rabbit anti-p35 monoclonal antibody (1:2000 dilution, Biocarta, San Diego, CA) and finally developed with chemiluminescence (Amersham, Piscataway, NJ).

2.4. Effects of p35 expression, caspase inhibitors and antioxidants on cell injury induced by hypoxia/reoxygenation

Cardiomyocytes were infected with Ad2/CMVp35 (10, 100 MOI) or Ad2/CMVEV (100 MOI) for 6 h and maintained under normal culture conditions for an additional 24 h. These cells were deprived of serum and glucose, placed in a hypoxic chamber ($<1\% O_2$, 5% CO$_2$, 37°C) for 90 min, and then returned to normoxic conditions (21% O$_2$, 5% CO$_2$, 37°C) in DMEM for an additional 15 h. Separate groups of non-infected cardiomyocytes were continuously treated during hypoxia/reoxygenation with the non-selective pan-caspase inhibitor, ZVAD-fmk (100 μM; Enzyme Systems, Livermore, CA) or the selective caspase 3 inhibitor DEVD-CHO (100 μM; Sigma–Aldrich, St Louis, MO). ZVAD-fmk and DEVD-CHO are peptide (acyloxy) methylketones. N-acetyl-L-cysteine (NAC, 1 mM; Sigma–Aldrich), an antioxidant, was added into the culture medium of additional groups of non-infected cardiomyocytes 3 h before the initiation of hypoxia. Cell viability, caspase activity, apoptosis, and cellular ROS levels were measured.

2.5. Measurement of caspase activity

Caspase 8 activity was measured using the ApoAlert Caspase Colorimetric Assay Kit (Clontech) in cardiomyocytes harvested with 0.25% (v/v) trypsin–EDTA 4 h after the initiation of reoxygenation, and was then
normalized to the total protein levels measured using the Bradford kit from Pierce (Rockford, IL). Caspase 3 activity was measured using the Apo-ONE™ Homogeneous Caspase-3/7 Assays kit (Promega, Madison, WI).

2.6. Measurement of ROS

Dichlorohydrofluorescein diacetate (DCFDA; Molecular Probes, Eugene, OR) was used to measure intracellular ROS levels. Thirty hours following infection with Ad2/CMVp35 or Ad2/CMVEV (100 MOI), cardiomyocytes in 96-well plates were incubated with DCFDA (30 μM) for 30 min and then subjected to hypoxia/reoxygenation. In separate groups of cells, NAC (1 mM) was added to the culture medium 3 h before the initiation of hypoxia. DCFDA fluorescence levels were measured prior to hypoxia, or 5, 15, 30, and 60 min after the initiation of reoxygenation using a fluorimeter (Molecular Devices, Sunnyvale, CA). The excitation and emission wavelengths were set at 488 and 530 nm, respectively. Data (mean±S.E.M., n=3) are expressed as arbitrary units of DCFDA fluorescence, or the rate of increase in DCFDA fluorescence per minute over a defined period time after the initiation of reoxygenation.

2.7. Measurement of the activity of antioxidant enzymes

Total superoxide dismutase (SOD) activity was determined using the Bioxytech SOD-525 kit (OXIS Health Products, Portland, OR), as outlined by the manufacturer. Copper/Zinc (Cu/Zn) SOD activity was measured in chloroform–ethanol extracted samples in which manganese (Mn) SOD was absent. Glutathione peroxidase (GPX) activity was measured using the Bioxytech cGPx-340 kit (OXIS Health Products). The enzymatic activity was normalized to the total protein levels, as measured using the Bradford kit (Pierce).

2.8. Detection of apoptosis

Cardiomyocytes were fixed in 3.7% (v/v) formaldehyde. Terminal deoxynucleotidyl transferase nick end-labeling (TUNEL) was performed using the ApoTACS in situ-TACS Blue TUNEL assay kit (R&D Systems, Minneapolis, MN). Nucleosome-sized DNA fragments were labeled with streptavidin–horseradish peroxidase and then reacted with fluorescein-conjugated anti-streptavidin antibody (Molecular Probes). The cell nuclei were counterstained with DAPI. Apoptotic nuclei were visualized and photographed at ×100 magnification using a microscope with an attached video camera. The number of TUNEL-positive cells was presented as a percentage of the total number of tropomyosin-positive cardiomyocytes.

2.9. Measurement of cell viability and lactate dehydrogenase activity

Cell viability was measured using the CellTiter96 Aqueous One kit (Promega), as outlined by the manufacturer. The cellular level of 3-[4,5-dimethylthiazol-2-yl-5]-[3-carboxymethoxyphenyl]-2-[4-sulfophenyl]-2H tetrazolium (MTS), an indicator of mitochondrial function of living cells, was linearly proportional to the number of viable cardiomyocytes (r>0.98). To assess the cytoplasm membrane integrity, lactate dehydrogenase (LDH) activity in the supernatant was measured using the TOX-7 cytotoxicity kit (Sigma–Aldrich).

2.10. Statistical analysis

Data are expressed as mean±S.E.M. The number of samples examined is indicated by ‘n’ in the figure legends. Data were analyzed by ANOVA, followed by a modified Student’s t-test. Unpaired Student’s t-test was used in experiments involving two groups only. A probability value of less than 0.05 was considered statistically significant.

3. Results

3.1. Adenoviral-mediated gene transfer into cardiomyocytes

Forty-eight hours following infection with Ad2/CMVp35 (10 and 100 MOI), cardiomyocytes expressed recombinant p35 protein, as measured by immunoblotting (Fig. 1A). Infection with Ad2/EGFP at a MOI of 10, 30, 100, and 300, resulted in transduction of 33±3%, 71±2%, 95±1%, and 100% of neonatal rat cardiomyocytes, respectively, as determined by light microscopy. These results indicate that adenoviral vectors are capable of transducing cardiomyocytes efficiently. In addition, compared to time-matched controls, infection with Ad2/CMVp35 up to a MOI of 1000 did not reduce cell viability, as measured by the MTS assay (Fig. 1B).

3.2. Effects of p35 on hypoxia/reoxygenation-induced increase in caspase activity

Hypoxia/reoxygenation increased the activities of caspases 3 and 8 in cardiomyocytes by approximately 70% and 60%, respectively, compared to time-matched controls (Fig. 2). As expected, either the pan-caspase inhibitor ZVAD-fmk or the selective caspase 3 inhibitor DEVD-CHO (100 μM) diminished the increase in caspase 3 activity following hypoxia/reoxygenation, but the activity of caspase 8 was inhibited by the pan-caspase inhibitor ZVAD-fmk only. Infection with Ad2/CMVp35 (100 MOI) completely inhibited the hypoxia/reoxygenation-induced
increase in the activities of caspases 3 and 8, whereas Ad2/CMVEV had no effect (Fig. 2). There was a trend that the antioxidant NAC also reduced caspase 3 activation, but the reduction did not reach statistical significance. These results suggest that p35 inhibits activation of caspases in cardiomyocytes during hypoxia/reoxygenation, as effectively as the pan-caspase inhibitor ZVAD-fmk.

3.3. Effects of p35 on cellular ROS levels following hypoxia/reoxygenation

Prior to hypoxia/reoxygenation, DCFDA fluorescence levels in cardiomyocytes under control conditions, treated with NAC (1 mM), or infected with Ad2/CMVEV or Ad2/CMVP35, were 16.0±0.4, 10.5±0.1, 15.8±0.2, and 15.7±0.2 (arbitrary units; n=3), respectively. Due to the transfer of the cells from the hypoxic chamber to the DCFDA fluorescence measurement apparatus, which was set up in a normoxic environment, we could only start to reliably determine the DCFDA fluorescence levels approximately 5 min after the initiation of reoxygenation. Fig. 3A shows that following hypoxia/reoxygenation, DCFDA fluorescence levels in non-infected cardiomyocytes were significantly higher at all time-points examined, compared
decreased the rate of increase in DCFDA fluorescence per minute, as effectively as NAC, whereas infection with Ad2/CMVEV had little effect. These results suggest that, in addition to its inhibitory effect on caspase activity, p35 is also capable of reducing cellular ROS levels, as measured by DCFDA fluorescence, in cardiomyocytes following hypoxia/reoxygenation. It is likely that the ability of p35 to inhibit cellular ROS levels contributes to its effective protection of cardiomyocytes against hypoxia/reoxygenation injury.

3.4. Effects of p35 on cellular SOD and GPX activity following hypoxia/reoxygenation

To assess the mechanism underlying the effect of p35 on cellular ROS levels following hypoxia/reoxygenation, we measured the activity of SOD and GPX. Infection with either Ad2/CMVEV or Ad2/CMVp35 did not affect the activity of Cu/Zn SOD, Mn SOD, and GPX (Fig. 4). These results are consistent with the notion that p35 functions directly as an antioxidant by mopping up free radicals [35].

3.5. Effects of p35 on cardiomyocyte apoptosis following hypoxia/reoxygenation

The TUNEL assay was used to detect apoptotic nuclei following hypoxia/reoxygenation in cardiomyocytes. In cells maintained under normal culture conditions (control), 2% of the nuclei stained TUNEL positive (Fig. 5). Hypoxia/reoxygenation caused no apparent cell detachment. However, the nuclei of approximately 18% of the cardiomyocytes were TUNEL positive and showed a pattern of DNA condensation and fragmentation that was characteristic of apoptosis (Fig. 5B). Whereas Ad2/CMVEV had no effect, the number of cardiomyocytes that stained TUNEL positive was reduced to 4% by infection with Ad2/CMVp35 (p35) or Ad2/CMVEV (EV) and their non-infected counterparts (H/R) were subjected to hypoxia/reoxygenation. Separate groups of non-infected cells were continuously treated with ZVAD-fmk (ZVAD, 100 μM) or DEVD-CHO (DEVD, 100 μM). NAC (1 mM) was added to additional non-infected cells 3 h prior to the initiation of hypoxia. The activities of caspase 3 (A) and caspase 8 (B) were measured. Only caspase 3 activity was measured in cells treated with NAC. Data are expressed as percentage of time-matched controls (mean±S.E.M., n≥3). * and ** indicate P<0.01, 0.001, and 0.0001, respectively, compared to H/R or EV.

to their counterparts maintained under normal culture conditions. This increase in DCFDA fluorescence was significantly inhibited by the antioxidant NAC (1 mM). Whereas Ad2/CMVEV had no effect, infection with Ad2/CMVp35 significantly inhibited the increase in DCFDA fluorescence following reoxygenation (Fig. 3A). We also analyzed the rate of increase in DCFDA fluorescence per minute over the period from the 6th to the 15th minute and from the 16th to the 60th minute after the initiation of reoxygenation (Fig. 3B, C). Infection with Ad2/CMVp35 decreased the rate of increase in DCFDA fluorescence per minute, as effectively as NAC, whereas infection with Ad2/CMVEV had little effect. These results suggest that, in addition to its inhibitory effect on caspase activity, p35 is also capable of reducing cellular ROS levels, as measured by DCFDA fluorescence, in cardiomyocytes following hypoxia/reoxygenation. It is likely that the ability of p35 to inhibit cellular ROS levels contributes to its effective protection of cardiomyocytes against hypoxia/reoxygenation injury.

3.6. Effects of p35 on hypoxia/reoxygenation-induced cell death

Compared to time-matched normoxic controls, hypoxia/reoxygenation reduced cell viability by approximately 40%, as measured by the MTS assay. Infection with Ad2/CMVp35 (100 MOI) 30 h prior to hypoxia/reoxygenation effectively inhibited cell death, whereas Ad2/CMVEV at the same dose had no effect (Fig. 6A). Although the antioxidant NAC also significantly reduced
cell death, the extent of the reduction by NAC was smaller than Ad2/CMVp35. There was a trend that the pan-caspase inhibitor ZVAD-fmk decreased cell death, but the reduction did not reach statistical significance ($P=0.14$). Treatment with the caspase 3 selective inhibitor DEVD-CHO had no effect. The protection of cardiomyocytes by p35 was confirmed by determining the LDH activity. Compared to time-matched controls, hypoxia/reoxygenation increased LDH activity in the culture medium by 85%, indicative of the disruption of the cytoplasm membrane and cell death. Infection with Ad2/CMVEV did not significantly affect LDH activity, nor did treatment with ZVAD-fmk or DEVD-CHO (Fig. 6B). In contrast, infection with Ad2/CMVp35 significantly reduced LDH activity following hypoxia/reoxygenation in a dose-dependent manner (Fig. 6B). Treatment with NAC also significantly reduced LDH activity following hypoxia/reoxygenation. However, the extent of the decrease of LDH activity by NAC was smaller than Ad2/CMVp35. Taken together, adenovirus-mediated expression of p35 effectively inhibited cell death induced by hypoxia/reoxygenation. NAC also partially inhibited cell death following hypoxia/reoxygenation, whereas pharmacological caspase inhibitors were ineffective.
4. Discussion

4.1. Protection of cardiomyocytes against hypoxia/reoxygenation injury by adenovirus-mediated expression of p35

The effect of p35 expression on hypoxia-induced cell injury has been assessed previously in cardiomyocytes from a transgenic mouse model. An adenoviral vector expressing the Cre-recombinase gene was used to turn on the expression of p35 prior to subjecting the cells to prolonged anaerobic conditions for 24 or 48 h in the presence of serum and glucose [33]. These authors observed that prolonged hypoxia-induced apoptosis was markedly reduced in cardiomyocytes from transgenic mice expressing p35. However, recent studies suggest that other components of ischemia/reperfusion significantly contribute to cardiomyocyte injury. Depletion of serum and glucose, an important component of ischemia, is sufficient to initiate apoptosis in cardiomyocytes [5]. Glucose uptake and glycolysis protect cardiomyocytes from hypoxia-induced apoptosis [36]. Hypoxia-induced cardiomyocyte apoptosis may require reoxygenation or a pH shift [19,37]. In this study, we simulated ischemia/reperfusion by depleting serum and glucose and subjecting the cells to reoxygenation. We demonstrated that adenovirus-mediated gene transfer of p35 effectively protected the cardiomyocytes from simulated ischemia/reperfusion injury.

4.2. Inhibition of caspase activity by p35

The ability of pharmacological caspase inhibitors to rescue cardiomyocytes from ischemia/reperfusion injury remains controversial [5–9,19]. In this study, as expected, the caspase 3 selective inhibitor DEVED-CHO abolished the increased activity of caspase 3, an effector/executioner caspase, but had no effect on the elevated activity of caspase 8, an initiator caspase [1]. However, DEVED-CHO did not reduce either apoptosis, as measured by the TUNEL assay, or total cell death, as determined by the MTS and LDH assays. The pan-caspase inhibitor ZVAD-fmk completely inhibited hypoxia/reoxygenation induced activation of caspases 3 and 8, as did adenoviral-mediated expression of p35. However, whereas ZVAD-fmk inhibited simulated ischemia/reperfusion-induced apoptosis by 50% only, adenoviral-mediated expression of p35 reduced apoptosis by 90%. Furthermore, the inhibition of apoptotic morphology by ZVAD-fmk did not fully translate into reduction of cell death, as the decrease in total cell death by ZVAD-fmk did not reach statistical significance. These observations suggest that following simulated ischemia/reperfusion, cardiomyocytes cannot be effectively rescued by inhibiting the activity of the caspases alone. Our results are consistent with the notion that inactivation of caspases and inhibition of the apoptotic morphology does not prevent cell death induced by some proapoptotic stimuli.
Fig. 5. Effects of p35 expression and pharmacological agents on cell apoptosis induced by hypoxia/reoxygenation. Neonatal rat cardiomyocytes infected with Ad2/CMVp35 (p35) or Ad2/CMVVEV (EV) and their non-infected counterparts (H/R) were subjected to hypoxia/reoxygenation. Separate groups of non-infected cells were continuously treated with ZVAD-fmk (ZVAD, 100 μM) or DEVD-CHO (DEVD, 100 μM). NAC (1 mM) was added to additional non-infected cells 3 h prior to the initiation of hypoxia. Cell apoptosis was determined using the TUNEL assay. (A) shows characteristic nuclear staining of cardiomyocytes maintained under control conditions (control), subjected to hypoxia/reoxygenation (H/R), or infected with Ad2/CMVp35 and then subjected to H/R (p35; magnification ×400). The green fluorescence depicts tropomyosin staining, while the arrow points to a TUNEL-positive nucleus. (B) shows representative fields of apoptotic nuclei following different treatment (magnification ×100). The quantitative results are summarized in (C). Data are expressed as a percentage of approximately 200 cardiomyocytes counted for each sample (mean±S.E.M., n=3). *, **, and *** indicate P<0.05, 0.001, and 0.0001, respectively, compared to H/R or EV.
including H$_2$O$_2$, growth factor withdrawal, ultraviolet radiation, and Bax, which initiate mitochondria-dependent apoptosis [10,12]. The effective inhibition of caspase activity, apoptosis, and total cell death by adenovirus-mediated expression of p35 may imply that p35 acts at an upstream step of activation of caspases.

4.3. Inhibition of ROS production by p35

Cellular ROS levels are elevated following ischemia/reperfusion, which contributes to reperfusion damage [13,38–40]. ROS generated by hypoxia/reoxygenation causes accumulation of ceramide, release of cytochrome c and precursors of caspases from the mitochondria, and apoptosis [17–21,41]. ROS also directly causes DNA strand breaks and the cell with the damaged DNA is then targeted for apoptosis by p53 through upregulation of Bax [15,16]. Bax, a mammalian cell death protein that targets mitochondrial membranes, can induce mitochondrial damage and cell death even when caspases are inactivated [42]. Antioxidants and metallothioneins have been shown to decrease cardiac cell apoptosis and total cell death following ischemia/reperfusion, highlighting the role of ROS in ischemia/reperfusion injury [22–25]. Recent studies suggest that p35 reduces cellular ROS levels in S. frugiperda cells [35]. In this study, we demonstrated that adenovirus-mediated expression of p35 in cardiomyocytes effectively reduced ROS levels following reoxygenation, as did treatment with the antioxidant NAC. Whereas the effect of p35 expression on the activity of catalase, the main intracellular scavenger of hydroperoxide, was not assessed in this study, p35 expression had no effect on the activity of SOD and GPX in cardiomyocytes. Although the effect of p35 on catalase and other intracellular scavenging systems cannot be ruled out, these results are consistent with the notion that p35 functions directly as an antioxidant by mopping up free radicals [35]. NAC also reduced cardiomyocyte apoptosis and cell death following hypoxia/reoxygenation. These results suggest that the ability of p35 to reduce cellular ROS levels may contribute to the protection of cardiomyocytes against hypoxia/reoxygenation injury.

It was noted that, despite complete inhibition of the increase in cellular ROS levels, inhibition of caspase 3 activity by NAC did not reach statistical significance. NAC reduced hypoxia/reoxygenation-induced apoptosis and cell death by 30% only, whereas p35 protected the vast majority of the cardiomyocytes subjected to hypoxia/reoxygenation. We speculate that reduction of cellular ROS levels and resultant interception of ROS-initiated apoptotic signaling prior to or at the mitochondrial stage of suicidal death by p35 may account for part of the protective effect only. In this study, caspase 8 activity was increased following hypoxia/reoxygenation. One potential mechanism for the activation of caspase 8 may involve the Fas ligand (FasL)/Fas system [1]. Cardiac expression of both FasL and Fas is upregulated by ischemia/reperfusion [3,43,44]. Upon binding by FasL, Fas initiates the signaling cascade for recruitment and activation of caspase 8, an initiator caspase. Activated caspase 8 not only acts on the mitochondria but also directly activates the effector/executioner caspases such as caspase 3, which creates a
though the relative contribution of the ability of p35 to act as an antioxidant and a caspase inhibitor to the inhibition of cell death remains unclear, both properties appear to be important for the protection of cardiomyocytes. It will be interesting to evaluate whether combining a pharmacological pan-caspase inhibitor (ZVAD-fmk) and an antioxidant (NAC) will mimic the effective protection of cardiomyocytes by p35 against hypoxia/reoxygenation injury. Further studies are also required to elucidate the role of the FasL/Fas pathway in mitochondria-dependent and -independent apoptosis following ischemia/reperfusion.

In summary, we demonstrated that adenovirus-mediated expression of p35 inhibited cardiomyocyte cell death induced by simulated ischemia/reperfusion. It is likely that the effective protection of cardiomyocytes against simulated ischemia/reperfusion injury by p35 involves its ability to inhibit caspase activity and reduce cellular ROS levels. These studies may help explore the therapeutic potential of antiapoptotic proteins and compounds for the treatment of ischemic heart disease and congestive heart failure which are associated with apoptosis and loss of cardiomyocytes [2,45].

Acknowledgements

We thank Geoff Y. Akita, Yuxia Luo, Ronald K. Scheule, Karen A. Vincent, Nelson S. Yew, and Yunxiang Zhu for their advice. Our thanks also go to the Viral Vector Production Group at Genzyme and especially to Denise Pratt for the supply of adenoviral vectors.

References


Fig. 6. Effects of p35 expression on cell viability (A) and LDH activity (B) induced by hypoxia–reoxygenation. Neonatal rat cardiomyocytes infected with Ad2/CMVp35 (p35) or Ad2/CMV EV (EV) and their non-infected counterparts (control) were subjected to hypoxia/reoxygenation. Separate groups of non-infected cells were continuously treated with ZVAD-fmk (ZVAD, 100 μM) or DEVD-CHO (DEVD, 100 μM). NAC (1 mM) was added to additional non-infected cells 3 h prior to the initiation of hypoxia. Cell viability was measured using the MTS assay. LDH activity in the supernatant was also determined using the TOX-7 kit. Data are expressed as percentage of the time-matched controls under normoxic conditions (mean±S.E.M., n=6). *, **, and *** indicate P<0.05, 0.01, and 0.0001, respectively, compared to H/R or EV.

shortcut for apoptosis [1]. Whereas ROS-mediated mitochondrial damage, apoptosis, and cell death were inhibited by the antioxidant NAC, this shortcut can be disrupted by inhibition of caspase, but not by reduction of ROS. On the other hand, in the absence of the caspase activity, mitochondrial damage is still sufficient to induce cell death, evident by the ineffectiveness of caspase inhibitors. Al-


