Serpin Protein CrmA Suppresses Hypoxia-Mediated
Apoptosis of Ventricular Myocytes

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Background—In this study, we ascertain whether caspase 8 activation and mitochondrial defects underlie apoptosis of ventricular myocytes during hypoxia. As an approach to circumvent the potential shortcomings surrounding the limited permeability and short half-life of the synthetic peptide inhibitors designed to block caspase activation, we constructed a replication-defective adenovirus encoding the serpin caspase inhibitor protein CrmA to ensure efficient and continual inhibition of caspase 8 activity during chronic hypoxia.

Methods and Results—In contrast to normoxic cells, oxygen deprivation of postnatal ventricular myocytes for 24 hours resulted in a 9-fold increase ($P<0.05$) in apoptosis as determined by Hoechst 33258 staining and nucleosomal DNA laddering. Moreover, hypoxia provoked a 1.5-fold increase ($P<0.01$) in caspase 8–like activity. Furthermore, hypoxia provoked perturbations to mitochondria consistent with the mitochondrial death pathway, including permeability transition pore (PT) opening, loss of mitochondrial membrane potential ($\Delta V_m$), and cytochrome c release. Importantly, CrmA suppressed caspase 8 activity, PT pore changes, loss of $\Delta V_m$, and apoptosis but had no effect on hypoxia-mediated cytochrome c release. Furthermore, Bongkrekic acid, an inhibitor of PT pore, prevented hypoxia-induced PT pore changes, loss of $\Delta V_m$, and apoptosis but had no effect on hypoxia-mediated cytochrome c release.

Conclusions—To our knowledge, we provide the first direct evidence for the operation of CrmA as an antiapoptotic factor in ventricular myocytes during prolonged durations of hypoxia. Furthermore, our data suggest that perturbations to mitochondria including PT pore changes and $\Delta V_m$ loss are caspase-regulated events that appear to be separable from cytochrome c release. (Circulation. 2001;103:1984-1991.)

Key Words myocytes • apoptosis • hypoxia • gene therapy

Programmed cell death or apoptosis has been documented in cardiac disease conditions including myocardial infarction,1 hypoxia,2–4 ischemia-reperfusion5 in parts of the conducting system, and more recently in individuals with end-stage heart failure.7,8 Because ventricular myocytes lose their capacity to proliferate shortly after birth, the loss of potentially viable cells through an apoptotic process may have profound clinical implications with respect to cardiac structure and function, given the limited and meager ability of the heart for repair after injury.

Genetic studies in the nematode Caenorhabditis elegans have identified ced-3, ced-4, and ced-9 genes, which encode proteins crucial for regulating cell number during worm development. Mammalian homologues of ced-3, ced-9, and ced-4 include the interleukin-1 converting enzyme, the bcl-2 gene, and Apaf-1, respectively.9 To date, more than 14 different but related interleukin-1 converting enzyme family members have been identified, collectively known as caspas (reviewed in Reference 10).

Recently, a mitochondrial pathway for apoptosis has been proposed (reviewed in Reference 11).11 Perturbations to mitochondria resulting in the loss of $\Delta V_m$ may occur from the opening of a large multiprotein conductance channel referred to as permeability transition pore (PT). The PT pore, which is composed in part of the adenine nucleotide translocator porin/VDAC and other mitochondrial membrane proteins, presumably opens in response to pro-death signals, leading to mitochondrial swelling, dissipation of $\Delta V_m$, and cytochrome c release.12

However, whether hypoxia-induced caspase activation induces mitochondrial defects leading to loss of $\Delta V_m$ and apoptosis in ventricular myocytes is unknown and has not been formally tested. It is equally unknown whether PT pore opening and $\Delta V_m$ loss are caspase regulated and events necessary for cytochrome c release during hypoxia-mediated apoptosis of ventricular myocytes.

Therefore, in this study, we determined whether mitochondrial defects including PT pore opening, loss of $\Delta V_m$, and cytochrome c release occur during hypoxia-mediated apoptosis of neonatal ventricular myocytes. We further determined whether adenovirus-mediated delivery of the cow pox cytokine response modifier protein (CrmA) would suppress...
caspase activation, mitochondrial defects, and apoptosis of ventricular myocytes during hypoxia.

Methods

Cell Culture
Ventricular myocytes were isolated from 1- to 2-day-old Sprague-Dawley rats and submitted to primary culture in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (Life Technologies, Inc), as previously reported.13 The media was removed 24 hours later and replaced with serum-free DMEM supplemented with 1 mg/mL insulin, 5 mg/mL transferrin, 1 nmol/L Na2SeO4, 1 nmol/L LiCl, 25 mg/mL ascorbic acid, and 1 nmol/L thyroxine, as previously reported. 13

Recombinant Adenovirus
The AdCrmA adenovirus consisting of the 1.4 kb crmA cDNA (generously provided by D. Pickup, Duke University) was generated by homologous recombination in human embryonic 293 kidney cells, as previously reported.14 Twenty-four hours after myocyte isolation, cells were infected in serum-free DMEM with AdCrmA virus at a titer of 20 plaque-forming units per cell for 4 to 6 hours. The viral suspension was removed, and cells were incubated for an additional 20 hours in serum-free DMEM before experimentation.15

Hypoxia
Postnatal ventricular myocytes were subjected to hypoxia for 24 hours in an air-tight chamber in culture medium that was continually gassed with 95% N2–5% CO2. These conditions were modeled after our own preliminary studies 4 as well as previously published work by Long et al16 and Tanaka et al,2 demonstrating that this duration of hypoxia was sufficient to trigger apoptosis of neonatal ventricular myocytes.

Assays of Apoptosis
Genomic DNA was subjected to gel electrophoresis as previously reported.17 Nuclear morphology was assessed by Hoechst 33258 dye (Molecular Probes) as reported.15 Cells were visualized with an Olympus AX70 epifluorescence microscope as described previously.5 15 Cells were analyzed from at least 3 independent myocyte isolations, counting ≥200 cells for each condition tested.

Detection of Caspase 8
The proteolytic activation of caspase 8-like protease activity was determined by fluorogenic assay with the substrate Iso-Glu-Thr-Asp-7-amino-4-trifluoromethyl coumarin (IETD-AFC) for caspase 8 with 40 mg of cardiac lysate protein. Hydrolysis of the IETD substrate was followed at 405 nm for 60 minutes. Data are expressed as mean±SEM from 3 independent myocyte cultures with replicates of 3 for each condition (activity is expressed as nmol AFC/mg per minute) (Clontech).

Immunofluorescence Microscopy
After interventions, myocytes were incubated with 0.1 mmol/L MitoTracker Red, (chloromethyl-rosamine CMX-Ros, Molecular Probes) for the detection of intact respiring mitochondria. Fixed cells were incubated with 1 mg/mL of a murine antibody directed toward cytochrome c (Pharmingen) followed by anti-mouse conjugated fluorescein IgG (1:150) (Roche Diagnostics) and detected with an Olympus Fluoview confocal microscope.4

Western Blot Analysis
For detection of CrmA, the Western blot filter was probed with a rabbit antibody directed toward CrmA (generously provided by Dr David Pickup, Duke University). The cytoplasmic S-100 fraction was prepared by methods previously described.17 Appropriate control experiments were used to ensure purity and completeness of separation of mitochondrial and S-100 fractions.4 Proteolytic cleavage of caspase 8 was detected with an antibody directed toward the 40-kDa and 20-kDa proteolytic fragments of caspase 8 (Pharmingen). Bound proteins were visualized with enhanced chemiluminescence reagents (Amersham).

Mitochondrial Membrane Potential $\Delta\Psi_m$ and MPT
Mitochondrial membrane potential $\Delta\Psi_m$ was monitored with the potential sensitive dyes JC-1 dye (1 $\mu$mol/L, 5.5',6.6'-tetraethyl-
benzimidazolylcarbocyanine iodide) or TMRM (50 nmol/L, tetramethyl rhodamine methyl ester perchlorate, Molecular Probes). A fluorescence distribution curve of individual cells was generated for each condition tested. Fluorescent intensities were then compared with each respective control group. Data are expressed as mean±SEM percent reduction of DCm from control for JC-1. To monitor mitochondrial PT, ventricular myocytes were loaded with 5 mmol/L calcein-acetoxymethylester (calcein-AM, Molecular Probes) in the presence of 2 to 5 mmol/L cobalt chloride to quench the cytoplasmic signal.18

Statistical Analysis

Multiple comparisons between groups were determined by 1-way ANOVA. An unpaired 2-tailed Student’s t test was used to compare mean differences between the control and hypoxic groups. Differences were considered to be statistically significant at a level of P<0.05.

Results

In contrast to normoxic control cells, a 9-fold increase (P<0.05) in apoptotic nuclei by Hoechst 33258 dye for nuclear morphology was observed in cardiac myocytes subjected to hypoxia (Figure 1, A and B). Moreover, in contrast to normoxic control cells, a significant increase in nucleosomal DNA laddering by gel electrophoresis was observed in hypoxic cells–indicative of cells undergoing apoptosis (Figure 1C).

Because the activation of caspase 8 can reportedly influence the mitochondrial pathway leading to apoptosis, we ascertained whether the zymogen form of caspase 8 was processed during hypoxia. As shown by Western blot analysis (Figure 2), hypoxia resulted in a significant increase in proteolytic cleavage of caspase 8 compared with normoxic control cells. Moreover, to verify that hypoxia-induced processing of caspase 8 was associated with an increase in caspase 8 activity, we monitored caspase 8 activity with the fluorogenic substrate IETD-AFC. The proteolytic cleavage of IETD-AFC yields a fluorescence signal that can be used to assess the level of caspase 8–like activity in myocytes during hypoxia. As shown in Figure 3, a 1.5-fold (P<0.01) increase in caspase 8–like activity was observed in hypoxic myocytes compared with normoxic control cells. To test the involvement of caspase 8 during hypoxia-mediated apoptosis, we generated a replication-defective adenovirus encoding the serpin protein CrmA from cow pox virus, a known inhibitor of caspase 8 activity.19 After adenoviral infection, Western blot analysis confirmed the presence of the 38-kDa CrmA protein in ventricular myocytes infected with the AdCrmA but not in cells infected with a control virus lacking the crmA c-DNA insert (Figure 4). Importantly, hypoxia-induced activation of caspase 8–like activity was suppressed in myocytes expressing CrmA but not in cells infected with a control virus, verifying that CrmA was functionally active in hypoxic myocytes compared with normoxic control cells. To test the involvement of caspase 8 during hypoxia-mediated apoptosis, we generated a replication-defective adenovirus encoding the serpin protein CrmA from cow pox virus, a known inhibitor of caspase 8 activity.19 After adenoviral infection, Western blot analysis confirmed the presence of the 38-kDa CrmA protein in ventricular myocytes infected with the AdCrmA but not in cells infected with a control virus lacking the crmA c-DNA insert (Figure 4). Importantly, hypoxia-induced activation of caspase 8–like activity was suppressed in myocytes expressing CrmA but not in cells infected with a control virus, verifying that CrmA was functionally active in hypoxic myocytes and sufficient to inhibit caspase 8 activity (Figure 3). Importantly, CrmA suppressed hypoxia-mediated apoptosis of ventricular myocytes, as evidenced by the reduction in Hoechst 33258–positive nuclei and nucleosomal DNA laddering (Figure 1) compared with cells infected with control virus. The data establish that AdCrmA is functionally active and sufficient to suppress hypoxia-mediated apoptosis of ventricular myocytes.
Activation of the Mitochondrial Death Pathway
To test whether hypoxia provokes mitochondrial release of cytochrome c, we monitored the subcellular localization of cytochrome c in situ (green fluorescence) and the mitochondrial dye MitoTracker (red fluorescence) by confocal microscopy. As shown in Figure 5A, in normoxic cells, cytochrome c staining was punctate and completely localized to mitochondria, as evidenced by the appearance of yellow fluorescence from overlay of the cytochrome c (green) and MitoTracker (red) fluorescent signals (Figure 5C). In contrast, cytochrome c staining in hypoxic cells was predominantly localized to the cytoplasm, as evidenced by the appearance of green staining bands at the level of the sarcomeres—consistent with the release of cytochrome c by mitochondria (Figure 5, D and F). This suggests that hypoxia alone is sufficient to provoke the release of cytochrome c by mitochondria. Interestingly, CrmA did not prevent hypoxia-induced cytochrome c release (Figure 5, G and I). These observations were confirmed by Western blot analysis, which showed that cytochrome c was absent in the S-100 fraction of normoxic cells but readily detectable in the S-100 fraction of hypoxic cells in the presence or absence of CrmA (Figure 5), verifying the confocal data for cytochrome c.

Hypoxia Induces Mitochondrial Defects Consistent With Loss of $\Delta \Psi_m$ and PT Changes
Because changes in mitochondrial function resulting from opening of the mitochondrial PT pore and loss in $\Delta \Psi_m$ can reportedly activate the apoptotic pathway, we next ascertained whether hypoxia-mediated apoptosis is associated with a loss in $\Delta \Psi_m$. For these experiments, we used the potential sensitive dye JC-1 to monitor changes in $\Delta \Psi_m$.17,20 As shown in Figure 6A, mitochondria of normoxic cells stained exclusively red with JC-1 dye, indicating a high membrane potential and polarized state. To verify that the red emission of the JC-1 dye accurately represented mitochondria with high $\Delta \Psi_m$, we treated ventricular myocytes with carbonyl cyanide 3-chlorophenylhydrazone (CCCP) to dissipate the mitochondrial H$^+$ ion transmembrane potential as a positive control for loss of $\Delta \Psi_m$. In contrast to control cells, cells treated with the mitochondrial uncoupler displayed a marked reduction in red fluorescing mitochondria, verifying that dissipation of mitochondrial membrane potential results in a reduction in JC-1 red fluorescence (Figure 6B). This validates the utility of JC-1 dye for monitoring changes in $\Delta \Psi_m$ in ventricular myocytes during hypoxia. In contrast to normoxic controls cells, ventricular myocytes subjected to hypoxia displayed a reduction in red fluorescing mitochondria, indicating a reduction in $\Delta \Psi_m$ (Figure 6, A versus C; E, histogram). Identical results were obtained with the mitochondrial potential sensitive dye TMRM, demonstrating hypoxia induced a loss of $\Delta \Psi_m$, supporting the JC-1 data (R.M. Gurevich and L.A. Kirshenbaum, unpublished data). Importantly, hypoxia-induced loss of JC-1 red fluorescence was
suppressed in myocytes expressing CrmA (Figure 6D, 

$P < 0.001$).

We next determined whether hypoxia promotes mito-
ochondrial PT pore opening. For these experiments, ventricle myocytes were loaded with the membrane-permeant dye calcein AM in the presence of cobalt chloride. The loss of green fluorescence in mitochondria can be used to monitor changes in mitochondrial membrane permeability and as an index of PT pore opening.\(^18\) As shown in Figure 7, in contrast to normoxic cells, which displayed punctate green staining mitochondria—indicative of intact mitochondrial membranes—hypoxic myocytes displayed a marked reduction in green fluorescing mitochondria consistent with PT pore opening. Importantly, ventricular myocytes expressing CrmA and subjected to hypoxia were similar to normoxic controls with respect to calcein staining mitochondria (Figure 7C), suggesting that CrmA-inhibitable caspases influence mito-

chondrial PT pore changes during hypoxia. To verify that the loss of mitochondrial calcein staining during hypoxia was a result of PT pore changes, we examined whether Bongkrekic acid (50 $\mu$mol/L), a known inhibitor of the PT pore,\(^{20-22}\) would prevent the loss of mitochondrial calcein staining during hypoxia. In contrast to control cells subjected to hypoxia, cells pretreated with Bongkrekic acid before the onset of hypoxia were indistinguishable from normoxic control cells with respect to green fluorescing mitochondria (Figure 7D), confirming that hypoxia influenced mitochondrial permeability changes consistent with PT pore opening. Interestingly, hypoxia-induced cytochrome c release was not prevented by Bongkrekic acid (R.M. Gurevich and L.A. Kirshenbaum, unpublished data), suggesting that PT pore opening and loss of $\Delta \Psi_m$ may be dissociable from cytochrome c release. Importantly, treatment of ventricular myocytes with Bongkrekic acid suppressed apoptosis of ventricular myocytes comparable
Discussion

To our knowledge, the experiments described here provide the first indication that the cow pox virus protein CrmA is sufficient to suppress hypoxia-induced apoptosis of ventricular myocytes. Moreover, our data indicate that hypoxia-mediated apoptosis of ventricular myocytes involves mitochondrial defects consistent with PT pore opening, loss of $\Delta \Psi_{m}$, and cytochrome c release. Another important feature of our study is the observation that CrmA and Bongkrekic acid suppressed hypoxia-induced mitochondrial defects and apoptosis without affecting cytochrome c release, suggesting that perturbations to mitochondria other than cytochrome c release may be important for induction of apoptosis during hypoxia.

The notion that caspases play a critical role in the proximal and distal signaling events leading to apoptosis (reviewed in Reference 23) is substantiated by studies in which either genetic ablation or inhibition of caspases with synthetic peptide inhibitors zYVAD-fmk or zDVED-fmk have been shown to prevent caspase activation in different in vitro and in vivo models of apoptosis. However, although synthetic caspase inhibitors appear efficacious in preventing caspase activation and apoptosis after short exposures to pro-death signals, their limited permeability and half-life has questioned their therapeutic usefulness for suppressing apoptosis after extended exposures to pro-death signals, such as prolonged hypoxia. Therefore, to circumvent this limitation, we generated a replication-defective adenovirus encoding the cow pox virus protein CrmA to ensure uniform and continual inhibition of caspase 8 in ventricular myocytes subjected to hypoxia. Although CrmA has been suggested to inhibit other

Figure 7. Hypoxia provokes PT pore opening. Mitochondrial PT pore was monitored in ventricular myocytes with calcein-AM dye in presence of cobalt chloride. Onset of PT is marked by loss of green fluorescence from mitochondria. A, Normoxic control myocytes; B, hypoxia; C, myocytes infected with adenovirus encoding CrmA and subjected to hypoxia; and D, myocytes subjected to hypoxia in presence of Bongkrekic acid. Bar=30 $\mu$m.

Figure 8. Hypoxia-induced apoptosis is suppressed with Bongkrekic acid. Apoptosis of ventricular myocytes is suppressed by PT inhibition with Bongkrekic acid. Ventricular myocytes were stained with Hoechst 33258 dye for nuclear morphology. A, Normoxic control cells (CNTL); hypoxia (HYPX); and myocytes subjected to hypoxia in presence of Bongkrekic acid (HYPX+BA). B, Histogram for data shown in A. Data are expressed as mean±SEM, with replicates of 3 for each condition from 2 independent experiments. Bar=5 $\mu$m.
caspases, it has recently been shown that CrmA is a selective caspase inhibitor with the greatest specificity for inhibiting caspase 1 and caspase 8. Because the role of caspase 1 in apoptosis has been questioned, caspase 8 has been determined to be the primary apoptotic caspase inhibited by CrmA. Toward this goal, we show that after adenovirus infection, CrmA protein was stably expressed and functionally active in myocytes after 24 hours of hypoxia, verifying the utility of this approach for suppressing apoptosis after extended durations of hypoxia.

Mitochondrial cytochrome c release has been suggested to play a key role in the apoptotic signaling pathway. Presumably, cytochrome c, through its interaction with Apaf-1, pro-caspase 9, and dATP, results in the activation of distal caspases and apoptosis. The mode by which cytochrome c is released by mitochondria is unknown, but several recent reports suggest that it may be related to changes in PT pore and $\Delta \Psi_m$. The fact that CrmA and Bongkrekic acid prevented hypoxia-induced loss of $\Delta \Psi_m$ yet had no apparent effect on cytochrome c release suggests that in ventricular myocytes, hypoxia-induced cytochrome c release may not be mutually dependent or obligatorily linked to loss of $\Delta \Psi_m$. This is in agreement with our previous studies and recent reports indicating that cytochrome c release can occur through a caspase-independent mechanism. The fact that CrmA and Bongkrekic acid suppressed hypoxia-induced apoptosis independent of cytochrome c release would strongly suggest that cytochrome c alone may not be sufficient for induction of apoptosis of ventricular myocytes during hypoxia.

Because changes in $\Delta \Psi_m$ can reportedly promote the release of proapoptotic factors by mitochondria, it is tempting to speculate that CrmA suppresses apoptosis through a mechanism that prevents the release of such factors. An alternative explanation holds that CrmA may suppress apoptosis by directly or indirectly inhibiting the activation of caspases or caspase substrates downstream of cytochrome c.

Nevertheless, under the conditions tested, the experiments described here provide the first evidence that mitochondrial defects leading to loss of $\Delta \Psi_m$ during hypoxia are mediated by caspases inhibitable by CrmA. Furthermore, adenovirus-mediated delivery of CrmA is sufficient to suppress apoptosis of ventricular myocytes during hypoxia.

Notwithstanding, it must be stated that developmental differences between the neonatal and adult myocardium are likely, and the current results may not predict the impact of the mitochondrial death pathway in the adult myocardium during acute or chronic phases of hypoxia without formal testing. However, our preliminary findings indicate that CrmA is sufficient to suppress hypoxia-mediated apoptosis of adult ventricular myocytes (K.M. Regula and L.A. Kirshenbaum, unpublished data), substantiating the findings of the present study. Therefore, genetic interventions designed to prevent caspase activation and apoptosis in patients with chronic oxygen deprivation associated with congenital cardiac defects or reduced coronary flow may prove beneficial in preserving cardiac cell function.

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References
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