Adrenomedullin Protects Against Myocardial Apoptosis After Ischemia/Reperfusion Through Activation of Akt-GSK Signaling

Hang Yin, Lee Chao, Julie Chao

Abstract—Adrenomedullin (AM) is a potent vasoactive peptide and plays an important role in cardiovascular function. In this study, we delivered the AM gene locally into the heart, using a catheter-based technique to investigate the signaling mechanism mediated by AM in protection against cardiomyocyte apoptosis induced by acute ischemia/reperfusion. After adenovirus-mediated gene delivery, highly efficient and specific expression of luciferase, green fluorescent protein, or recombinant human AM was identified in the left ventricle. Delivery of the AM gene 5 days before ischemia/reperfusion attenuated myocardial apoptosis identified by in situ dUTP nick-end labeling and DNA laddering, and the effect was blocked by the AM antagonist human calcitonin gene–related peptide (CGRP 8 to 37). AM gene transfer increased phosphorylation of Akt and glycogen synthase kinase (GSK-3β) but reduced GSK-3β and caspase-3 activities in the heart. The effects of AM on GSK-3β and caspase-3 activities were blocked by CGRP (8-37) and by adenovirus containing dominant-negative Akt (DN-Akt). Furthermore, in cultured cardiomyocytes, AM also attenuated apoptosis induced by hypoxia/reoxygenation, which was accompanied by increased phospho-GSK-3β but reduced GSK-3 and caspase-3 activities. GSK-3 and caspase-3 activities were both blocked by Ad.DN-Akt and lithium, whereas only caspase-3 was inhibited by its inhibitor Z-VAD. The effects of AM on anti-apoptosis and promoting cell viability were blocked by DN-Akt but not by constitutively active Akt, lithium, or Z-VAD. These results indicate that AM protects against cardiomyocyte apoptosis induced by ischemia/reperfusion injury through the Akt-GSK-caspase signaling pathway. (Hypertension. 2004;43:109-116.)

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Adrenomedullin (AM), a potent vasodilator, has been identified in tissues relevant to cardiovascular and renal function. Abnormal AM levels have been reported in a number of human diseases. For example, plasma AM levels are increased in patients with essential hypertension, cardiac hypertrophy, heart failure, and renal disease. Thus, elevated AM production could be a biological attempt to compensate for cardiac and renal damage. Transgenic mice overexpressing the AM gene have decreased mean blood pressure with no change in heart rate. Nitric oxide synthase (NOS) inhibition normalized blood pressure in these transgenic mice, supporting a role for nitric oxide (NO) in mediating the effects of AM. Furthermore, embryos of AM-knockout mice die at midgestation with extreme hydrops fetalis and cardiovascular abnormalities. These findings implicate important roles of AM in the function and development of the cardiovascular system. AM may act as an autocrine/paracrine modulator in the process of cardiac remodeling and apoptosis in the heart. Recent studies showed that AM inhibited cardiac myocyte apoptosis through a cAMP-dependent mechanism in vitro and protected against cerebral ischemic injury in vivo. These combined results suggest a potential protective role of AM in apoptosis during myocardial injury.

Recent findings indicate that the serine-threonine kinase Akt is a powerful survival signal leading to anti-apoptosis and cell survival, and it lies at the crossroads of activated multiple apoptotic stimuli during myocardial injury. On nuclear translocation, Akt phosphorylates a variety of transcription factors and other regulatory proteins. One of the downstream signaling protein kinases of Akt is glycogen synthase kinase (GSK-3), which represents a convergence site of multiple signaling pathways involved in cell fate. Phosphorylation at serine 21 (α-isofrom) or serine 9 (β-isofrom) of GSK-3 leads to inhibition of its activity and reduces apoptosis. Inactivation of GSK-3 by Akt may thus contribute to antiapoptotic effects of phosphotidal inositol-3 kinase (PI-3K)/Akt signaling. This is evidenced by the finding that overexpression of a dominant-negative mutant of GSK-3β prevents apoptosis after inhibition of PI-3K. In addition,
several studies indicate that activation of PI-3K/Akt suppresses caspase-3 activation and DNA fragmentation in a variety of cell lines.\textsuperscript{15–17} However, the role of the interaction and signaling pathway among Akt, GSK-3, and caspase-3 in myocardial apoptosis has not been explored.

Using a systemic gene transfer approach, we previously reported that human AM gene delivery attenuates hypertension and protected against cardiac hypertrophy, fibrosis, and renal damage in hypertensive rats.\textsuperscript{18–21} In this study, we established a catheter-based technique for local AM gene transfer to investigate the effects of AM on apoptosis and cell survival and its signaling mechanism in protection against ischemia/reperfusion (I/R)-induced myocardial apoptosis in vivo and in vitro. Our findings show that the Akt/GSK3/caspase-3-dependent pathway plays an important role in mediating the protection of AM against myocardial apoptosis.

\section*{Methods}

\subsection*{Replication-Deficient Adenoviral Vectors}

Adenoviral vectors harboring human AM (Ad.CMV-AM), green fluorescent protein (Ad.CMV-GFP), or Luciferase (Ad.CMV-Luc) cDNA under the control of the cytomegalovirus (CMV) enhancer/promoter or without a reporter gene (Ad.Null) were prepared as described.\textsuperscript{21} Adenoviruses containing dominant-negative mutant of Akt (Ad.DN-Akt) and constitutively active Akt (Ad.Myr-Akt) were kindly provided by Dr. Kenneth Walsh, St Elizabeth’s Medical Center in Boston.

\subsection*{Catheter-Based Gene Delivery}

Wistar rats (male; weight, 250 to 280 g; Harlan) were used in this study. The study complied with the standards for care and use of Laboratory Animals (National Academy of Sciences). Five days before coronary occlusion, gene delivery was performed with the use of a catheter-based strategy as described.\textsuperscript{22,23} Briefly, rats were anesthetized, intubated, and mechanically ventilated before surgery. The chest was entered through a left intercostal approach. A 24-gauge catheter (BD) containing 300 pfu/mL in PBS was advanced from the apex of the left ventricle to the site of the catheter, and the solution was injected. The clamp was maintained for 15 seconds. After injection, the exposed heart was monitored for 5 minutes for resumption of normal sinus rhythm. Mean arterial pressure (MAP) and electrocardiography were recorded throughout the experimental period.

\subsection*{Myocardial I/R Animal Model}

Acute myocardial I/R models were established as previously described.\textsuperscript{24} Briefly, a 6-0 polypropylene suture (Ethicon) was passed loosely around the left anterior descending (LAD) coronary artery near its origin. Once hemodynamics were stabilized, LAD occlusion was performed by tightening the suture loop for 30 minutes followed by 120 minutes of reperfusion. At the end of reperfusion, the ischemic regions were then removed for further analysis.\textsuperscript{24}

Rats were randomly divided into 6 groups. In the sham group, the chest was opened and injected with saline (control, n=8). The I/R control group (n=7) was also injected with saline. The third group received Ad.Null (n=7) and the fourth group received Ad.CMV-AM (n=8). The fifth group received Ad.CMV-AM together with administration of AM antagonist, human calcitonin gene-related peptide, CGRP(8-37), delivered intraperitoneally by osmotic minipump (Alzet) at 0.55 mg/kg per day (n=8).\textsuperscript{25} The sixth group was injected with combination of Ad.CMV-AM and Ad.DN-Akt (n=6).

\subsection*{In Situ Nuclear DNA Fragmentation and DNA Laddering}

DNA fragmentation was determined by means of a terminal deoxyxynucleotidyl transferase–mediated dUTP nick-end labeling (TUNEL) detection kit (Roche).\textsuperscript{24} The ratio of TUNEL-positive cardiomyocytes to the total number of cardiomyocytes was calculated. DNA laddering was analyzed as previously described.\textsuperscript{24} DNA fragments were separated by agarose gel electrophoresis and visualized under ultraviolet light.

\subsection*{In Vitro Cell Culture and Hypoxia/Reoxygenation}

Cardiomyocytes were isolated from the hearts of 2- to 3-day-old Wistar rats (Harlan) with an enzymatic technique, then cultured in DMEM/F-12 medium with 10% FBS. Cardiomyocyte origin was confirmed immunohistochemically using antibody to α-actin (Sarcomeric, Sigma). Cultured cells were growth-arrested in serum-free medium for 18 hours at 37°C before the experiments. Cells were transduced with Ad.CMV-AM, Ad.Null, Ad.Myr-Akt, and Ad.DN-Akt at MOI 50 for 12 hours followed by 12-hour hypoxia (95% N\textsubscript{2}, and 5% CO\textsubscript{2}) and 24-hour reoxygenation (95% O\textsubscript{2}, and 5% CO\textsubscript{2}). Before hypoxia/reoxygenation (H/R), cardiomyocytes were treated with 20 mmol/L LiCl, (GSK-3β inhibitor) for 30 minutes or 100 μmol/L Z-VAD (caspase-3 activity inhibitor) for 60 minutes. Apoptotic cardiomyocytes were identified by TUNEL staining (Roche), as previously described.\textsuperscript{26} Cell viability was determined by trypan blue eliminating assay.

\subsection*{GSK-3β Kinase and Caspase-3 Activity Assays}

Control and injured ventricular tissues were pulverized under liquid nitrogen and homogenized in ice-cold lysis buffer, as described previously.\textsuperscript{24} Caspase-3 activity in lysates was determined using a colorimetric caspase-3 assay kit according to the manufacturer’s instructions (Clontech). Samples were read at 405 nm in a microplate reader.

\subsection*{Western Blot Analysis}

Heart tissues and cultured cardiomyocytes were homogenized in the protein extraction buffer containing 10 mmol/L Tris, pH 7.4, 100 mmol/L NaCl, 0.1% SDS, 1% Triton X-100, 5 mmol/L EDTA, 2 mmol/L Na\textsubscript{2}VO\textsubscript{4}, 1:100 protease inhibitor cocktail (Sigma). Aliquots were resolved on SDS-PAGE. Proteins were transferred to a nitrocellulose membrane and then incubated with the primary antibodies that recognize Akt, phospho-Akt, phospho-GSK-3β, GSK-3β and cleaved caspase-3 (cell signaling) at 4°C overnight. Bound antibodies were detected by a secondary antibody conjugated to horseradish peroxidase and visualized by ECL chemiluminescence (NEN Life Science Products).

\subsection*{Immunohistochemistry}

Expression and localization of human AM in rat ventricles after gene delivery were identified immunohistochemically by antibody to human AM (1-52) (Phoenix Pharmaceuticals).

\subsection*{Statistical Analysis}

Data are expressed as mean±SEM and were compared between experimental groups with the use of 1-way ANOVA followed by Fisher protected least squares difference. Probability values of P<0.05 were considered statistically significant.

\section*{Results}

\subsection*{Catheter-Based Adenoviral Gene Transfer for Global Expression in Rat Heart}

Using a catheter-based gene transfer technique, the highest levels of luciferase activity were detected in the myocardium, but very low levels were found in the liver, lung, brain, and kidney (Figure 1A). Left ventricles transsected with Ad.CMV-GFP showed homogenous expression of green
fluorescence, whereas the Ad.Null-transfected myocardium showed little background fluorescence (Figure 1B). The expression and localization of human recombinant AM in the left ventricle was confirmed and identified immunohistochemically at 4 to 6 days after local gene transfer. No specific staining was found in the left ventricle of the Ad.Null-injected group (Figure 2).

Hemodynamic monitoring showed that both HR and MAP are comparable among the experimental and control groups at basal levels before and after local gene delivery. No differences in HR or MAP were observed among all groups. Administration of the AM antagonist, CGRP(8-37) in the AM-treated group also did not elicit any changes in HR or MBP (data not shown).

### AM Gene Delivery Attenuates Apoptosis in the Acute I/R Rat Model

Figure 3A shows representative apoptotic cardiomyocytes identified by TUNEL staining in the I/R-injured region. The ratio of TUNEL-positive cardiomyocytes to total number of cardiomyocytes in the Ad.CMV-AM group was significantly reduced as compared with the Ad.Null group (22.7±4.8% versus 43.4±6.1%, n=8 and 7, P<0.001). This beneficial effect was abolished by CGRP(8-37) (22.7±4.8% versus 40.9±5.8%, n=8 and 8, P<0.001) (Figure 3B). Results of TUNEL detection were in accordance with DNA fragmentation assay (Figure 3C). DNA laddering was not visualized in the sham-operated heart tissue, whereas I/R markedly increased DNA fragmentation. AM gene transfer abrogated I/R-induced DNA fragmentation, whereas the protective effect of AM was reversed by CGRP(8-37). These results indicate that expression of AM in the heart protects against I/R-induced cardiomyocyte apoptosis.

### Effects of AM on Akt, GSK-3β, and Caspase-3 in I/R Injury

Western blot analysis showed that Ad.CMV-AM delivery resulted in increased Akt and GSK-3β phosphorylation in the heart after I/R as compared with the controls, with or without delivery of Ad.Null. The effects of AM on Akt and GSK-3β phosphorylation were abolished by the AM antagonist CGRP(8-37) or Ad.DN-Akt (Figures 4A through 4C). Similarly, cleaved caspase-3, which is a downstream proapoptotic signal, was markedly reduced after AM gene delivery, whereas the inhibitory effect of AM on caspase-3 activation was abrogated by CGRP(8-37) and Ad.DN-Akt (Figures 4A and 4D). The immunoblot results were further confirmed by measurements of GSK-3β kinase and caspase-3 activities. AM gene transfer significantly reduced GSK-3β activities by 27.7% and caspase-3 activities by 33.3% compared with I/R controls (n=8 or 7, P<0.01). These effects were also blocked by CGRP(8-37) and Ad.DN-Akt (Figures 4E and 4F). These combined data indicate that Akt activation and inhibition of GSK-3β and caspase-3 activities mediate the protective effect of AM on cardiac apoptosis after I/R injury.

### Interactions of GSK-3β and Caspase-3 in H/R-Induced Cardiomyocyte Apoptosis In Vitro

To further investigate the signaling mechanisms mediating AM protection in myocardial apoptosis, we examined the effect of AM transduction on H/R-induced apoptosis in neonatal primary cultured cardiomyocytes. Cardiomyocyte apoptosis was effectively induced by H/R, and Western blot analysis showed that the transduction of Ad.CMV-AM in cardiomyocytes resulted in increased GSK-3β phosphorylation and reduced cleaved caspase-3 (Figures 5A through 5D). Ad.Null had no effect on either GSK-3 phosphorylation or caspase-3 activation under normoxic or H/R conditions. Both...
Effect of AM on H/R-Induced Apoptosis and Viability in Cultured Cardiomyocytes

Based on findings in vivo, we examined the effects of AM on signal events in relation to cardiomyocyte apoptosis and cell survival subjected to H/R (Figure 6). AM gene transduction significantly reduced cardiomyocyte apoptosis and increased viability compared with cells subjected to H/R (13.1 ± 2.9% versus 24.1 ± 4.7% and 74.3 ± 9.4% versus 58.2 ± 10.1%, n = 4, P < 0.01). There was no difference between H/R-treated cardiomyocytes with or without transduction with Ad.Null. The protective effect of AM was abolished by cotransduction with Ad.DN-Akt but not by Ad.Myr-Akt, LiCl or Z-VAD. Ad.Myr-Akt reduced the number of apoptotic cardiomyocytes and increased viable cells compared with control (10.6 ± 2.2% versus 24.1 ± 4.7% and 78.8 ± 10.7% versus 58.2 ± 10.1%, n = 4, P < 0.01). LiCl inhibited apoptosis and enhanced the viability of cardiomyocytes subjected to H/R injury in AM-transduced cells (10.3 ± 2.2% versus 24.1 ± 4.7% and 74.3 ± 9.5% versus 58.2 ± 10.1%, n = 4, P < 0.01). Similarly, Z-VAD also caused the same effects (11.3 ± 2.1% versus 24.1 ± 4.7% and 81.5 ± 8.7% versus 58.2 ± 10.1%, n = 4, P < 0.01), indicating the proapoptotic effect of caspase-3 after injury. Taken together, these combined results indicate that the effects of AM in protecting against myocardial apoptosis and improving cell survival is mediated by activation of Akt leading to inhibition of the GSK-3-caspase-3–dependent pathway.

Discussion

This is the first study to demonstrate that AM protects against myocardial apoptosis through activation of the Akt-GSK-mediated signaling mechanism. Using the I/R rat model, we showed that the expression of recombinant AM in cardiac cells activates Akt, leading to GSK-3 phosphorylation. Inactivation of GSK-3 by Akt-mediated phosphorylation led to inhibition of caspase-3 activation and thus attenuation of cell death. In cultured cardiomyocytes, AM also protects against H/R-induced apoptosis through the Akt-GSK-3-dependent pathway. These findings provide new insights into the role and signaling mechanisms mediated by AM in protection against I/R-induced cardiac injury and apoptosis and may have significance in the development of molecular targets for future therapeutic application.

Evidence from recent studies indicates a protective role of AM in heart failure, left ventricular remodeling after myocardial infarction, and myocyte hypertrophy. The mechanism by which AM exerts its effects on cardiomyocytes is not fully understood. A previous report showed that AM signaling depends on the combination of CRLR (calcitonin receptor-like receptor) and RAMP2 (receptor activity-
modifying proteins), resulting in activation of the AM receptor. In addition, AM and RAMP2 mRNA levels in the left ventricle were elevated in a rat heart failure model. This suggests that the effects of AM on cardiomyocytes were mediated by the CRLR/RAMP2 complex in the myocardium. We have recently shown that systemic delivery of the AM gene protects against cardiomyocyte apoptosis induced by I/R, which was accompanied by increased cardiac cGMP, an indicator of NO production, but not cAMP levels. NO has also been shown to increase AM receptor availability and AM signaling transduction in mesangial cells through a cGMP-dependent and cAMP-independent pathway. Based on these findings, it is likely that AM may protect against myocardial injury through the NO-cGMP signaling pathway.

In the present study, we showed that AM gene transfer had no effect on hemodynamic parameters throughout the experiment in normotensive rats. In addition, local AM gene delivery, together with infusion of CGRP(8-37) through

**Figure 4.** Effect of AM gene transfer on Akt, GSK-3β, and caspase-3 signaling in I/R myocardium. A, Representative Western blot of myocardial extracts from sham, I/R control, and I/R receiving Ad.Null, Ad.CMV-AM, Ad.CMV-AM/CGRP, or Ad.CMV-AM/DN-Akt (n=6 to 8). Phospho-Akt, Akt, phos-GSK-3β[Ser9], GSK-3β, and cleaved caspase-3 were identified. β-actin was loading control. Histograms show relative intensity of phospho-Akt (B), phosphor-GSK-3β (D) and cleaved caspase-3 (E), GSK-3β kinase activity assay (E), and caspase-3 activity assay (F), with corresponding methods. *P<0.01 vs other groups except sham.
osmotic minipumps, did not change the blood pressure and heart rate. In accord with our findings, a continuous infusion of AM peptide has also shown no effect on blood pressure of normotensive rats during a 4-week study. Moreover, a previous study showed that infusion of CGRP(8-37) through osmotic minipumps had no effect on the blood pressure of normotensive rats. Taken together, these results indicate that the cardioprotective effects of AM are independent of hemodynamic changes.

Akt has been shown to promote survival of cardiomyocytes in vitro as well as to protect against I/R-induced injury in the mouse heart. Our recent study showed that AM gene transfer inhibited I/R-induced myocardial apoptosis that was accompanied by activation of Akt and Bad and increased Bcl-2 levels, indicating a role of Akt-Bad-Bcl-2 signaling in AM-mediated cardiac protection against apoptosis. Moreover, GSK-3 is also a downstream target of Akt signaling. Overexpression of catalytically active GSK-3 induces apoptosis, whereas dominant-negative GSK-3 prevents apoptosis after inhibition of PI-3K. GSK-mediated signaling events in cardiomyocytes apoptosis are not clear. One recent report showed that ischemic preconditioning increased GSK-3β phosphorylation through a PI-3K/Akt–dependent pathway, suggesting that inhibition of GSK-3β is protective in myocardial precondition. Using an acute I/R animal model and cultured cardiomyocytes, we presented convincing evidence that expression of AM activates Akt, resulting in the inhibition of GSK activity in the antiapoptotic process. We showed

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**Figure 5.** Effect of AM gene transfer on GSK-3 and caspase-3 signaling in cultured cardiomyocyte transduced with Ad.CMV-AM in the absence of LiCl (20 mmol/L), Z-VAD (100 μmol/L), or cotransduced with Ad.Null, Ad.Myr-Akt, or Ad.DN-Akt before H/R. A, Representative photomicrographs of TUNEL (top) and phase control (bottom) in cardiomyocyte subjected to normoxia or H/R. Original magnification ×200. B, Western blot for phospho-GSK-3β(Ser9), GSK-3, and cleaved-caspase-3. β-actin was loading control. Representative data are from 4 independent experiments. Histograms show relative intensity of phosphor-GSK-3β (C) and cleaved caspase-3 (D), GSK-3β kinase assay (E), and caspase-3 activity assay (F), with corresponding methods (mean±SEM). *P<0.01 vs Ad.Null-transduced cardiomyocytes subjected to H/R.
that DN-Akt blocked AM-induced phosphorylation of GSK-3β, and inhibition of GSK-3 by LiCl promoted viability and reduced apoptosis in cardiomyocytes subjected to H/R. These results indicate that the protective effect of AM on apoptosis is mediated by Akt-dependent GSK-3β phosphorylation.

Caspase-3 is thought to be activated during the final step of the proapoptotic signaling pathway in many cell lines, and it represents a potential therapeutic target for suppression of cardiomyocyte apoptosis. In human cardiomyopathy, apoptosis of cardiac muscle cells is associated with release of cytochrome c and activation of caspase-3. I/R induces apoptosis in the myocardium, whereas inhibition of caspase activity has been shown to attenuate both I/R injury and apoptosis in the myocardium, whereas inhibition of caspase activity was not altered under this condition. In contrast, dominant-negative Akt and LiCl suppressed AM-induced phosphorylation of GSK-3β and caspase-3 activity. These results indicate a downstream event of caspase-3 activation after Akt-GSK signaling. Previous studies in human neuroblastoma cell lines showed that the increase in nuclear GSK-3β levels preceded activation of the caspase cascade, suggesting a potential role of GSK phosphorylation in suppressing caspase-activation.

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