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Omapatrilat enhances adrenomedullin's reduction of cardiomyocyte cell death☆

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Abstract

The objective of this study was to determine whether adrenomedullin, a vasodilator peptide, modulates the process of cell death in cardiomyocytes and whether its effect would be enhanced by the endopeptidase inhibitor omapatrilat, which reduces adrenomedullin degradation. Further, we sought to determine whether the effect of adrenomedullin involved an action to preserve mitochondrial transmembrane potential ($\Delta\Psi_m$). Cardiomyocytes in culture were treated with agents that interrupted the mitochondrial electron transport chain, inhibiting glycolysis and oxidative phosphorylation. Cell death was evaluated by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay and $\Delta\Psi_m$ was assessed by fluorescent microscopy. Cytochrome *c* loss from mitochondria and appearance in cytosol was determined by Western blotting. Potassium cyanide (KCN) plus deoxyglucose or antimycin A, for 24 h, produced significant ($p<0.01$) concentration-dependent reductions in cell viability or increases in cell death. Adrenomedullin reduced cell death produced in this manner and the effect of adrenomedullin was enhanced by treatment with omapatrilat. In contrast, there was no additional reduction in cell death by lisinopril treatment. Omapatrilat plus adrenomedullin reduced the KCN plus deoxyglucose-induced increase in cytosolic cytochrome *c*. A likely mechanism centers on the ability of adrenomedullin plus omapatrilat to prevent the decline in mitochondrial $\Delta\Psi_m$ produced by KCN plus deoxyglucose treatment. In summary, adrenomedullin plus omapatrilat limited the decline in mitochondrial $\Delta\Psi_m$ that accompanies interruption of mitochondrial metabolism and limited the extent of cell death in cardiomyocytes treated with KCN plus deoxyglucose or antimycin. Adrenomedullin plus the endopeptidase inhibitor omapatrilat may be a useful strategy to protect cardiomyocytes from cell death, in conditions associated with impairment of mitochondrial function.

Keywords: Adrenomedullin; Omapatrilat; Cardiomyocyte cell death; Cyanide antimycin A; Cytochrome *c*; Mitochondrial transmembrane potential

1. Introduction

Adrenomedullin, a member of the calcitonin gene-related peptide superfamily, is widely distributed in the body and induces its biological effects through both calcitonin and adrenomedullin receptors (Hinson et al., 2000; Kuwasako et al., 2004). The biological actions of adrenomedullin encompass the vascular effects of vasodilatation; autocrine and paracrine effects; diuresis; as well as a diverse spectrum of other effects so that adrenomedullin is recognized as an important cardiovascular regulatory peptide (Eto et al., 2003). The high expression of adrenomedullin receptors in the heart (Kitamura et al., 1993; Totsune et al., 2000) together with the ability of the heart to synthesize and secrete adrenomedullin (Romppanen et al., 1997; Shimokubo et al., 1996) lead to the proposal for a number of potentially important roles for adrenomedullin in cardiovascular disease. Adrenomedullin is part of the neurohumoral activation that characterizes hypertension and heart failure with adrenomedullin activation serving a compensatory role to lower blood pressure and promote diuresis (Jougasaki et al., 1995; Sumimoto et al., 1997). Thus adrenomedullin has been proposed to function as an endogenous cardiovascular protective peptide.

A cellular protective function for adrenomedullin was originally recognized in endothelial cells (Kato et al., 1997;

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Sata et al., 2000) subsequently demonstrated in tumour cells (Martinez et al., 2002; Oehler et al., 2001) and more recently found in the heart (Kato et al., 2003; Okumura et al., 2004; Yin et al., 2004). Data to support a cardioprotective effect of adrenomedullin relies on adrenomedullin infusion or delivery of adrenomedullin gene to the heart in myocardial ischemic reperfusion injury (Kato et al., 2003; Okumura et al., 2004; Yin et al., 2004). Whether exogenously administered adrenomedullin will protect the cardiomyocyte element of the heart and whether it will be effective in other models of cardiac cell injury is less clear. Further, intact animal studies cannot determine whether the effect of adrenomedullin is on coronary blood flow or ventricular remodeling or the cell type (fibroblasts, endothelial cells or vascular smooth muscle cells) involved. Thus studies are necessary in isolated cardiomyocytes to answer these questions.

Adrenomedullin degradation occurs through the action of metalloproteases, aminopeptidases and endopeptidases (Lewis et al., 1997). Neutral endopeptidases (NEP) are important enzymes involved in the degradation of kinins and are a major enzyme set inactivating natriuretic peptides (Erdos and Skidgel, 1989). In spontaneously hypertensive rats, the local concentration and action of adrenomedullin in the tissues appears to be differentially regulated by NEP (Jiang et al., 2004). Omapatrilat, a mercaptoacyl-based fused dipeptide mimetic has inhibitory activity against NEP and inhibits angiotensin converting enzyme (ACE) with similar K_i because of the similarity of the structure of the active sites of NEP and ACE (Robl et al., 1997). Vasopeptidase inhibition by omapatrilat increases adrenomedullin plasma levels whereas levels of the natriuretic peptides and cGMP are unchanged (Cataliotti et al., 2002; Maniu et al., 2002). Vasopeptidase inhibition potentiates the natriuretic actions of adrenomedullin (Lisy et al., 1998). Thus the objective of this study was to examine the hypothesis that exogenously administered adrenomedullin protects cardiomyocytes against cell death and that the NEP inhibitor omapatrilat will accentuate the effect of adrenomedullin.

2. Materials and methods

2.1. Cell cultures

Cardiomyocytes from embryonic ventricular cells were cultured from 7-day chick embryos from white Leghorn eggs using previously described methods (Rabkin and Kong, 2000). The protocol was approved by the University Committee on use of animals for research. Myocytes were maintained in culture in medium 818A (73% DBSK (NaCl 116 mM, $MgSO_4$ 0.8 mM, NaH_2PO_4 0.9 mM, dextrose 5.5 mM, $CaCl_2$ 1.8 mM, $NaHCO_3$ 26 mM), 20% M199, 2 or 6% fetal calf serum and 1% antibiotic–antimycotic (10,000 mg/ml streptomycin sulfate, 10,000 U/ml penicillin G sodium and 25 mg/ml amphotericin B) for 72 h prior to the experiment. The proportion of myocytes at this time was over 90% as verified by the proportion of cells showing spontaneous contraction or displaying muscle specific markers (myosin) on immunohistologic examination.

2.2. Cell viability assay

Cell viability was assessed by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay, which is based on the ability of viable cells to reduce MTT from a yellow water soluble dye to a dark blue insoluble formazan product (Mosmann, 1983). Cardiomyocytes were grown in multiwell microtitre plates (Becton Dickinson, Lincoln Park, NJ, USA) for 72 h and then treated with various agents. After the appropriate time, MTT dye was added to cardiomyocytes and the plates were incubated at 37 °C for 4 h. Solubilization reagent was added and the absorbance was determined at 570 nm on a multiwell plate reader (BioRad model#3550, BioRad, Mississauga, Canada). Background absorbance, of medium in the absence of cells, was subtracted. There is a highly significant linear relationship between cell number and absorbance (Rabkin and Kong, 2000).

2.3. Biochemical techniques

Whole cell lysates and subcellular fractions were prepared from cells grown for 72 h in culture. For whole cell lysates, cells were lysed with hypotonic buffer (10 mM TrisCl, pH 7.4, 25 mM NaF, 2 mM Na_3VO_4, 1 mM $ZnCl_2$, 10 mM β-glycerol phosphate, 10 mM sodium pyrophosphate, 1% protease inhibitor cocktail (Sigma Chemical Co. St. Louis, Mo, USA) and homogenized by passage through a 26G needle. Mitochondrial fractions were obtained (Li et al., 2003). Cells were lysed with hypotonic buffer and spun at 600 g for 10 min at 4 °C to remove nuclei and unlysed cells (pellet). Supernatant was then spun at 15,000 *g* for 5 min at 4 °C to separate mitochondria (pellet) from cytosol. The mitochondrial pellet was resuspended in RIPA buffer (150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate, 50 mM Tris, pH 8). This method yields a highly enriched mitochondrial fraction with little contamination, as ascertained by electron microscopy or assessment of mitochondrial enzyme activity, and demonstrated by characteristic mitochondrial respiration and ATP formation (Botla et al., 1995; Li et al., 2003). We have further verified that this mitochondrial preparation was rich in mitochondrial markers and did not have detectable cytosolic contamination (Kong et al., 2005).

2.4. Western blots

Cellular fractions were prepared as outlined above. Equivalent amounts of protein, assessed by Bradford protein assay, were loaded on a 12% polyacrylamide gel for electrophoresis. The gel was then transferred to a nitrocellulose membrane and blocked with 5% BSA in Tris-buffered saline (TBS) overnight at 4 °C. The membrane was then washed with 0.3% TBS-Tween 20 (TBST) and reacted with a primary antibody and detected with a horseradish peroxidase-linked secondary antibody as previously described (Rabkin and Kong, 2000). Signals were detected using Renaissance chemiluminescence reagents on a chemiluminescent film. Densitometric analysis was performed

with the Scion Image program (Scion Corporation, Maryland, USA).

2.5. Microscopy

Cell morphology was examined using Wright Giemsa staining. Cells were grown on coverslips for 72 h. They were treated and then they were fixed in methanol for 15 s, and subsequently stained for 15 s each in eosin and methylene blue solutions.

Mitochondrial potential ($\Delta\Psi_m$) was measured in intact cells using a fluorescent probe. To examine for mitochondrial potential, cardiomyocytes were grown on coverslips for 72 h before experimentation, were washed briefly with PBS and then allowed to incubate in 0.1% 5,5′6, 6′-tetrachloro-1, 1′, 3,3′ tetraethylbenzimidazolylcarbocyaninine iodide (DePsipher^{TM}) reaction mix with stabilizer (Trevigen Inc, Gaithersburg, MD, USA) at 37 °C for 20 min. Cells were washed with pre-warmed reaction mix before microscopic examination. Cells were examined by a fluorescent microscope as described (Kong and Rabkin, 2003).

2.6. Materials

All culture media, fetal calf serum, antibiotics and antimycotics were obtained from GIBCO (Burlington, Ontario, Canada). Potassium cyanide and deoxyglucose were from Sigma Chemical Co. (St. Louis, Mo, USA). Adrenomedullin was from Rose Scientific Ltd (Mississauga, Ontario, Canada). Antimycin A was from Calbiochem (LaJolla, CA, USA). Omapatrilat was provided by Bristol Meyers Squib. DePsipherTM was from Trevigen Inc (Gaithersburg, MD, USA). Antibodies against cytochrome *c* were from BD Biosciences (Mississauga, Ontario, Canada). Chemiluminescence reagents were from Amersham (Piscataway, New Jersey, USA) and chemiluminescent film was from Kodak (Rochester, NY, USA).

2.7. Data analysis

The data are presented as the mean±1S.E.M. Hypothesis testing used one way analysis of variance with Kruskal–Wallis multiple-comparison test for comparison of between group data (Statistical package was NCSS 6.0). The null hypothesis was rejected if the probability of a Type I error was less than 5% ($p<0.05$).

3. Results

Cell death was induced using the combination of potassium cyanide (KCN) and deoxyglucose which respectively inhibit glycolysis and oxidative phosphorylation (Nelson and Cox 2000). Cell viability was assessed by the MTT assay. KCN plus deoxyglucose produced a significant ($p<0.01$) reduction in MTT absorbance (Fig. 1 inset). Considering the linear relationship between absorbance and cardiomyocyte cell number (Rabkin and Kong, 2000), KCN plus deoxyglucose, for 24 h, produced a significant ($p<0.01$) concentration dependent

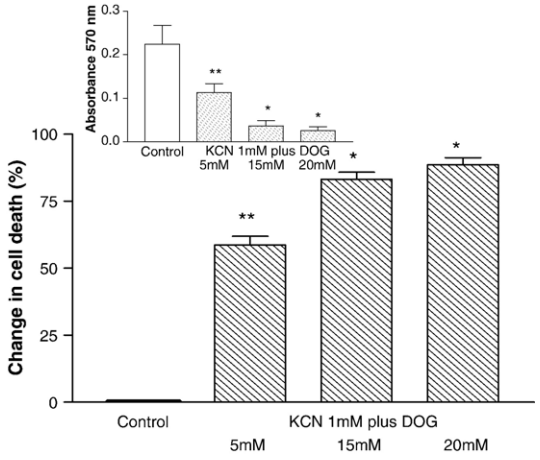


Fig. 1. *Cardiomyocyte viability after treatment with KCN and deoxyglucose as assayed by the MTT assay.* Cardiomyocytes were seeded in multiwell microtitre plates at 30,000 cells per well. After 72 h, cells were treated with KCN plus deoxyglucose for 24 h. MTT dye was added to each well for the last 4 h of treatment. The reaction was stopped and the absorbance (optical density) was determined at 570 nm. Background absorbance of medium in the absence of cells was subtracted. The results are presented as the mean±S.E.M. for control ($N=10$) or 1 mM KCN plus deoxyglucose either 5 mM ($N=10$), 15 mM ($N=4$) or 20 mM ($N=4$) for 24 h. The insert shows the absorbance. The change in absorbance which is the change in cell death is shown as % control. Absorbance values are shown in the insert. Hypothesis testing showed a significant (ANOVA; $p=0.0011$) difference with pairwise comparison of KCN plus deoxyglucose from control being significantly different (*$p<0.05$, **$p<0.01$).

reduction in cell viability or increase in cell death (Fig. 1). KCN 1 mM plus deoxyglucose 5 mM produced a 62.7±3.3% increase in cell death compared to control. Increasing the concentration of deoxyglucose to 15 mM and 20 mM produced respectively an 83.2±2.7% and 88.5±2.7% increase in cell death.

The concentration of KCN 1 mM plus deoxyglucose 5 mM was chosen for further examination because it was more likely modifiable while higher concentrations produce more cell death that might be less amenable to modification with interventions. Adrenomedullin at 10 nM produced a significant ($p<0.05$) reduction in cell death that was slightly greater with adrenomedullin 100 nM (Fig. 2). To determine whether the effects of adrenomedullin might be enhanced by treatment with omapatrilat, cardiomyocytes were co-treated with adrenomedullin and omapatrilat. The effect of adrenomedullin to reduce cell death induced by KCN plus deoxyglucose was significantly ($p<0.05$) enhanced by omapatrilat 100 nM or 1 μM. In contrast, these concentrations of omapatrilat alone were not associated with any alteration in MTT absorbance or cell viability (Fig. 2 inset).

To determine whether the effects of adrenomedullin might be similarly enhanced by the angiotensin converting enzyme inhibitor lisinopril, cardiomyocytes were treated with 1 mM KCN plus 5 mM deoxyglucose alone ($N=4$), in combination with 10 nM adrenomedullin ($N=4$) alone or with lisinopril 1 μM ($N=4$) or 100 nM ($N=4$) for 24 h. In contrast to the results in Fig. 2, there was no further reduction in KCN plus deoxyglucose-induced cell death over the effect produced by adrenomedullin alone (data not shown).

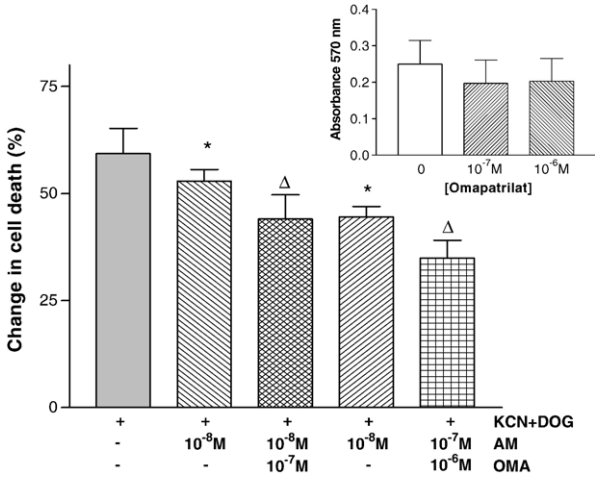


Fig. 2. *Cardiomyocyte viability after treatment with KCN and deoxyglucose is improved after treatment with adrenomedullin and omapatrilat*. Cardiomyocytes were seeded in multiwell microtitre plates at 30,000 cells per well. After 72 h, cells were treated with 1 mM KCN plus 5 mM deoxyglucose alone or in combination with 10 nM or 100 nM adrenomedullin, with or without 1 μM omapatrilat ($N=6$) for 24 h. Cell viability was assessed using the MTT assay. The results are presented as percent cell death (relative to control) and are the mean±S.E.M. Hypothesis testing showed a significant change from KCN plus deoxyglucose compared to control (*$P<0.05$) or KCN plus deoxyglucose plus adrenomedullin plus omapatrilat (Δ$P<0.05$). *Inset*: Cardiomyocyte viability was not altered by omapatrilat. Cardiomyocytes were seeded in multiwell microtitre plates at 30,000 cells per well. After 72 h, cells were treated with omapatrilat 100 nM ($N=4$) or 1 μM ($N=4$) or diluent (0) ($N=4$) for 24 h. Cell viability was assessed using the MTT assay. The results are presented as the mean±S.E.M. of the absorbance (optical density), determined at 570 nm, after the background absorbance of medium in the absence of cells was subtracted.

Next we sought to examine whether the effects of adrenomedullin plus omapatrilat were generalizable to more than the modes by which KCN and deoxyglucose inhibited mitochondrial function, so the effect of antimycin A, which inhibits the mitochondrial electron transport chain (Wolvetang et al., 1994) was assessed. Antimycin produced a significant ($p<0.01$) reduction in MTT absorbance (Fig. 3 inset) indicating a significant concentration dependent induction of cell death. Antimycin 1 μM, 10 μM, and 100 μM produced respectively a 44.9±8.2%, 59.2±8.0% and 78.9±6.8% increase in cell death compared to control (Fig. 3). Antimycin 1 μM was the concentration chosen for further examination because it produced a 44.9±8.23% increase in cell death, close to the IC_{50} for antimycin and likely more modifiable while higher concentrations produce more cell death that might be less likely to be subject to modification with other interventions.

The effect of each intervention on cell morphology was examined and both antimycin A or KCN plus deoxyglucose produced a dramatic change in cell structure after 24 h of treatment (Fig. 4). Cardiomyocytes treated with KCN 1 mM and deoxyglucose 5 mM showed changes of cell death, namely fragmentation of the nucleus with an increase in cytosolic vacuoles and disintegration of the cell membrane. Similar changes were produced by antimycin A which included marked cytoplasmic shrinkage, nuclear fragmentation and loss of the nuclear envelope. The nuclear damage in viable cells that received the combination of antimycin A plus deoxyglucose was profound.

The addition of adrenomedullin and omapatrilat caused a significant ($p<0.05$) decrease in cell death induced by antimycin 1 μM by about half, namely from 44.9±8.2% to 20.7±2.5% (Fig. 5A). To determine the potential for adrenomedullin plus omapatrilat to alter the more profound cytotoxicity of antimycin A plus deoxyglucose, cardiomyocytes were treated with antimycin A 1 μM plus deoxyglucose 5 mM. The addition of only omapatrilat 1 μM did not alter cell death but the combination of omapatrilat 1 μM plus adrenomedullin 1 μM to antimycin plus deoxyglucose-treated cells produced a modest but significant reduction in cell death from 88.4±4.9% to 74.8±2.6% (Fig. 5B).

The mitochondrial cell death pathway, an established mechanism of cell death, can be mediated through either the apoptotic or necrotic (oncotic) mode of death (Kroemer, 2003; Nieminen, 2003). The mitochondrial role in apoptotic cell death involves the release of mitochondrial constituents into the cytosol with subsequent activation of the caspase cascade (Regula et al., 2003). The classic substance released from mitochondria is cytochrome *c*. Cytochrome *c* was assessed in cytosolic and mitochondrial fractions of cardiomyocytes using cell fractionation methods shown to provide relatively pure separations (Kong et al., 2005). KCN plus deoxyglucose produced an increase in cytochrome *c* release into the cytosol (Fig. 6). Imaging analysis of the Western blot, calculating the sample integrated densities (number of pixels/area) of cytochrome *c* expressed relative to the integrated density of control, showed that KCN plus deoxyglucose produced a three-fold increase in cytochrome *c* release into the cytosol. Omapatrilat plus adrenomedullin produced a small but definite reduction in the amount of cytochrome *c* released into the cytosol after

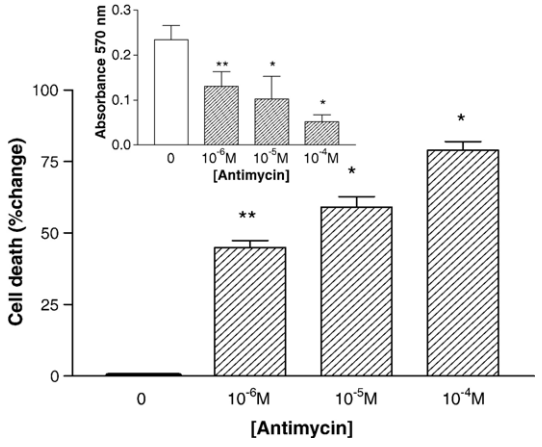


Fig. 3. *Cardiomyocyte viability after treatment with antimycin*. Cardiomyocytes were seeded in multiwell microtitre plates at 30,000 cells per well. After 72 h, cells were treated with antimycin A for 24 h. Cell viability was assessed using the MTT assay. The change in cell death is shown as percent of control. The results are presented as the mean±S.E.M. for control ($N=11$), antimycin 1 μM ($N=11$), antimycin 10 μM ($N=5$) or antimycin 100 μM ($N=5$). The inset shows the changes in absorbance. Hypothesis testing showed a significant ANOVA; $p=0.006$) difference as antimycin was different from control (*$p<0.05$, **$p<0.01$).

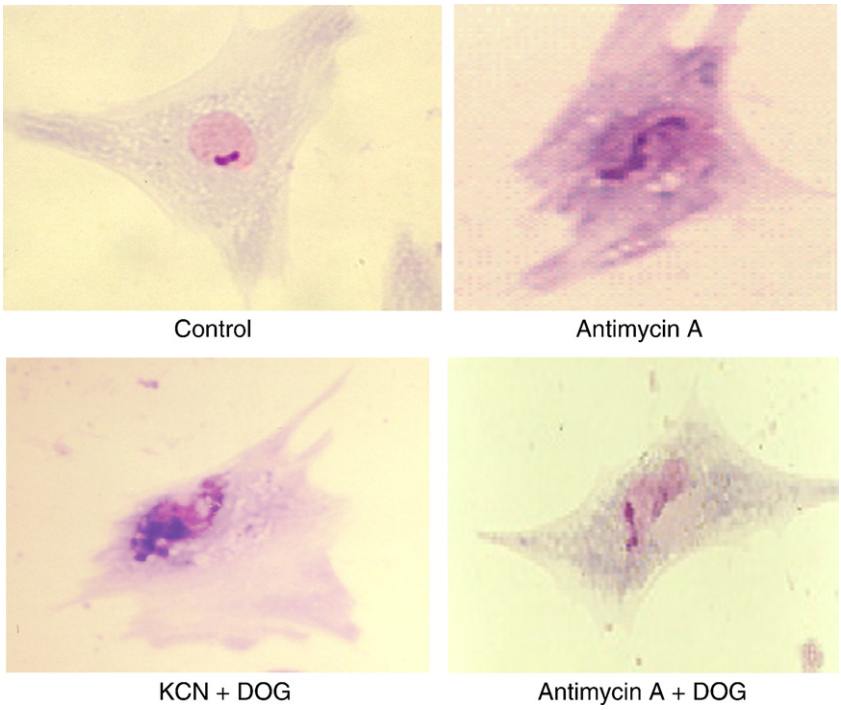


Fig. 4. *The cell morphology of cardiomyocytes after KCN plus deoxyglucose or antimycin*. Cardiomyocytes were grown in culture on coverslips for 72 h and then treated for 24 h with diluent (control), 1 mM KCN plus 5 mM deoxyglucose, antimycin 1 μM, or antimycin 1 μM plus 5mM deoxyglucose. Cells were stained with Wright-Giemsa stain and examined at 1600× magnification. Representative photomicrographs are shown.

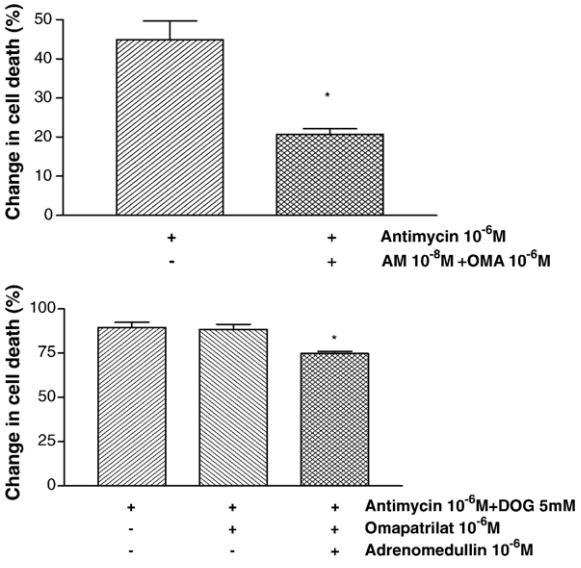


Fig. 5. *Cardiomyocyte viability after treatment with antimycin with or without deoxyglucose plus adrenomedullin and omapatrilat. Antimycin 1 μM*. Cardiomyocytes, after 72 h in culture, were treated with antimycin 1 μM plus the diluent for adrenomedullin and omapatrilat ($N=5$) or antimycin 1 μM plus 10 nM adrenomedullin and 10 μM omapatrilat ($N=5$) for 24 h. Cell viability was assessed using the MTT assay. The results are presented as the mean ± S.E.M. for the change in cell death (relative to control). *Antimycin 1 μM plus deoxyglucose 5 mM.* Cardiomyocytes, after 72 h in culture, were treated with the indicated combinations of antimycin 1 μM plus deoxyglucose 5 mM, adrenomedullin 1 μM, and omapatrilat 1 μM for 24 h ($N=5$). Cell viability was assessed using the MTT assay. The results are presented as the mean ± S.E.M. for the change in cell death (relative to control). Significant differences are noted from antimycin plus deoxyglucose (*$p<0.05$).

15 min of treatment and a 20% reduction from a 3.47-fold increase over control at 60 min.

Opening of high conductance permeability transition pores which initiates the onset of the mitochondrial permeability transition is a causative event leading to activation of necrotic or apoptotic cell death (Kim et al., 2003). The permeability transition pore is involved in the release of a number of discrete proapoptotic proteins, including cytochrome *c*, from mitochondria into the cytosol (Kroemer, 2003; Nieminen, 2003). Opening of the mitochondrial permeability transition pores reduces the $\Delta\Psi_m$, which can be used with certain caveats as a marker for the mitochondrial permeability transition pores and a sign of potential for cell death (Ly et al., 2003). Changes in $\Delta\Psi_m$ were evaluated through the use of a mitochondrial potential-dependent fluorescent stain and examined by

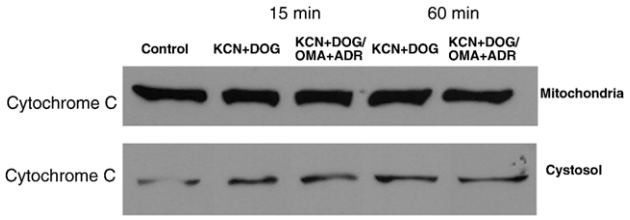
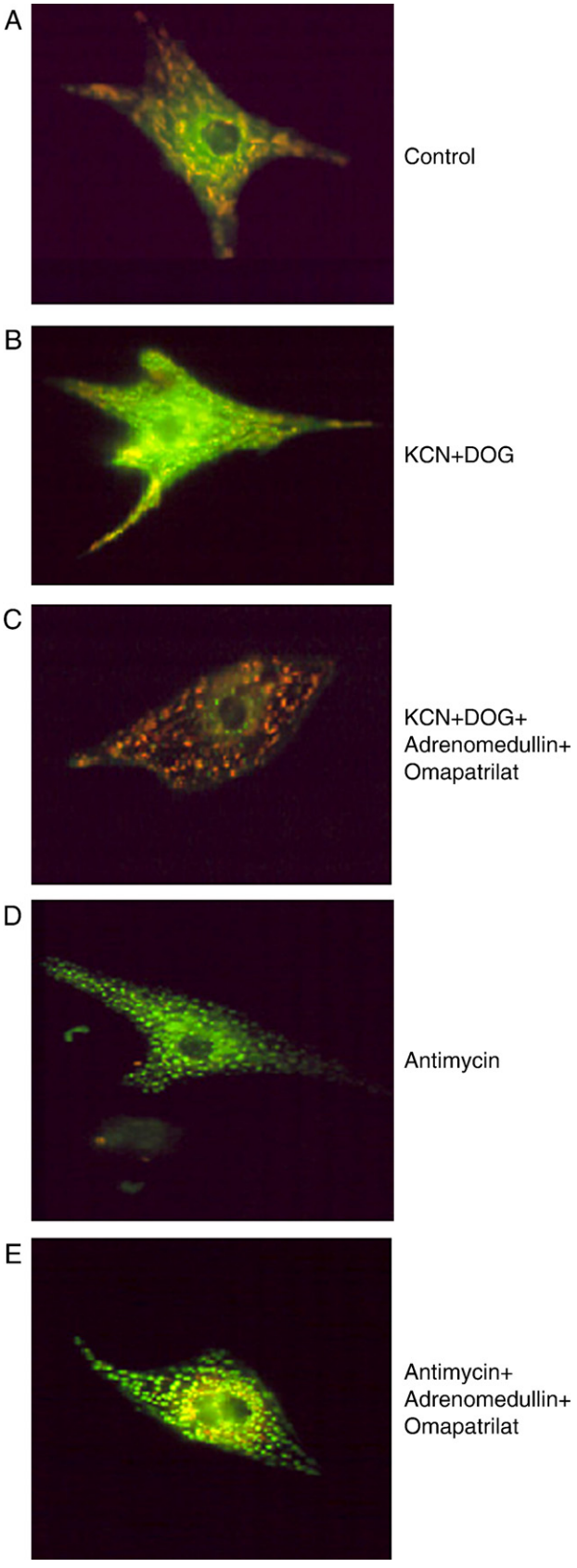


Fig. 6. *Adrenomedullin and omapatrilat modify the increase in cytosolic cytochrome c induced by KCN plus deoxyglucose.* Both cytosolic and mitochondrial fractions were separated from cells as described in Materials and methods. Representative Western blot immunoblotted with the mitochondrial marker cytochrome *c* in both cell fractions are shown.

fluorescent microscopy which assesses the overall state of mitochondrial $\Delta\Psi_m$ in a cell population (Smiley et al., 1991). At lower $\Delta\Psi_m$, less dye enters the mitochondria resulting in monomers that fluoresce green while at higher $\Delta\Psi_m$ the dye accumulates sufficiently in mitochondria to form aggregates that fluoresce red (Jiang et al., 1999). This is a useful method to measure the effects of agents to induce cardiomyocyte cell death through a mitochondrial pathway (Kong and Rabkin, 2003) and comparative studies with several different fluorochromes have shown that J-aggregates are the preferred dye for assessing $\Delta\Psi_m$ in cardiomyocytes (Mathur et al., 2000). There will always be some mitochondria with a lower potential (green monomers)even though the cell is healthy (Mathur et al., 2000). Representative photomicrographs depict control cells with red aggregates, indicating the presence of mitochondria with a high $\Delta\Psi_m$ (Fig. 7 Panel A). In contrast, KCN plus deoxyglucose produced a loss of $\Delta\Psi_m$, i.e. less red fluorescence and a strong uniform green fluorescence (panel D). A similar change, a picture of green fluorescence suggesting lower $\Delta\Psi_m$, was produced by antimycin A 100 mM (Fig. 7 Panels B and C). The addition of adrenomedullin and omapatrilat to both of these treatments appear to increase the number of red aggregates again, suggesting a beneficial salvage pathway for adrenomedullin and omapatrilat that prevents the collapse of mitochondrial membrane potential.



4. Discussion

This study demonstrates the novel finding of the capacity of an endopeptidase inhibitor to accentuate adrenomedullin-induced cardioprotection from cell death induced by interruption of mitochondrial metabolism and inhibition of mitochondrial electron transport. Our model of cardiomyocyte cell death involved inhibition of mitochondrial function by two different approaches. First, potassium cyanide and deoxyglucose which inhibit glycolysis and oxidative phosphorylation functions of mitochondria (Hasin et al., 1984) and second, antimycin A, an inhibitor of succinate oxidase and NADH oxidase, that have been conceptualized as Complex II of the series of protein electron carriers embedded in the inner membrane of mitochondria — the mitochondrial electron transport chain (Wolvetang et al., 1994). KCN plus deoxyglucose produce a rapid and almost 90% diminution of cellular ATP within 15 min followed by formation of intracellular gaps, actin cytoskeletal breakdown and increase in membrane macromolecule permeability (Watanabe et al., 1991). The mitochondrial action of KCN plus deoxyglucose is a composite of a classical action to produce necrotic (oncotic) cell death from interruption of the ability of the cell to maintain cellular ionic homeostasis as well as apoptotic cell death. It is likely that the mode of cell death

Fig. 7. *Adrenomedullin and omapatrilat reduce loss of mitochondrial potential induced by KCN plus deoxyglucose or antimycin*. Cardiomyocytes were plated on to coverslips and maintained in culture. After 72 h, cells were treated with 1 mM KCN plus 5 mM deoxyglucose, alone or combined with adrenomedullin 100 nM plus omapatrilat 100 nM for 24 h or antimycin 1 μM alone or with adrenomedullin 100 nM plus omapatrilat 100 nM for 24 h. Coverslips were then washed and stained with DePsipher stain, a mitochondrial potential-dependent fluorescent stain. Cells were examined and representative photomicrographs obtained at 1600× magnification.

induced by antimycin A is the same. Certainly caspase activation, the hallmark of apoptosis, occurs in ventricular cardiomyocytes after treatment with KCN plus deoxyglucose (Kaushal et al., 1997) and in other cell types after interruption of the mitochondrial electron transport chain by antimycin (Engelbrecht et al., 2004). We found that the morphologic alterations of cellular structure associated with KCN plus deoxyglucose or antimycin treatment are consistent with what we have previously demonstrated with agents that induce both apoptotic and oncotic cell death in these cells (Kong and Rabkin, 2000; Rabkin and Kong, 2000).

Omapatrilat enhanced the ability of adrenomedullin to limit the cell death induced by KCN plus deoxyglucose or antimycin A with or without deoxyglucose. Adrenomedullin was cardioprotective against cell death induced by interventions that inhibit mitochondrial function. While it may appear that the cardioprotection was somewhat greater for KCN plus deoxyglucose compared to antimycin A plus deoxyglucose, the amount of cell death was greater with the latter and it is more difficult to protect against a more potent interruption of cellular metabolic function. We focused on the mitochondrial pathway in the induction of cell death and to our knowledge this is the first demonstration of the ability of adrenomedullin to reduce cell death from stimuli that act almost exclusively at the mitochondrial level. The cardioprotective action of adrenomedullin against cell death induced by KCN plus deoxyglucose or antimycin is consistent with the ability of adrenomedullin to inhibit cardiac cell death in other models such as adriamycin toxicity (Tokudome et al., 2002), hypoxia/reoxygenation and coronary artery ischemia/reperfusion injury (Kato et al., 2003; Okumura et al., 2004; Yin et al., 2004).

Our demonstration that omapatrilat plus adrenomedullin modify myocardial cell death-induced by interruption of mitochondrial metabolism or the electron transport chain suggests a mitochondrial site of action for adrenomedullin. Adrenomedullin plus omapatrilat produced a slight diminution of KCN plus deoxyglucose-induced loss of cytochrome *c* from the mitochondria. An action on a Bcl-2 family member (Kato et al., 1997) would be anticipated to modify release of cytochrome *c* from the mitochondria. Other mechanisms operative through an upstream pathway such as a cAMP-dependent pathway utilizing protein kinase A (Tokudome et al., 2002) or an Akt phosphorylation pathway that may also involve glycogen synthase kinase 3β (Okumura et al., 2004; Yin et al., 2004) may also be involved.

While omapatrilat increased the effect of adrenomedullin on cardiomyocyte viability, lisinopril was without such an action demonstrating that the action of omapatrilat is specific to endopeptidase inhibition rather than ACE inhibition. In the rat model of acute myocardial infarction, omapatrilat improved survival, cardiac function and cardiac remodeling as well as reducing infarct size 24 h after myocardial infarction (Lapointe et al., 2002). While omapatrilat-treated animals had less myocardial apoptosis in the infarction border zone and in the remote area 4 weeks after myocardial infarction, a selective neutral endopeptidase inhibitor had no effect on apoptosis (Backlund et al., 2003). We did not find any effect of omapatrilat on the viability of isolated cardiomyocytes. This may be due to the ability of omapatrilat to blunt the increase in endothelin-1 or norepinephrine that occurs in the intact animal subjected to coronary artery ligation (Lapointe et al., 2002).

Mitochondria are not only a central site for the generation of metabolic energy, but they also participate as a major pathway leading to cell death (Adams, 2003). The mitochondrial membrane potential is a critical component of a functional mitochondria and healthy cell, as without it the driving force required for ATP synthesis would be absent. Both antimycin A and KCN plus deoxyglucose inhibit respiration and ATP production. The combination of KCN and deoxyglucose inhibits lactate production and respiration, which causes profound declines in cellular ATP levels in cardiomyocytes (Webster et al., 1994). Metabolic inhibitors such as KCN or antimycin also produce a marked loss of $\Delta\Psi_m$ in many cell types, including cardiomyocytes (Di Lisa et al., 1995; Wolvetang et al., 1994), which is associated with cell death (Kong and Rabkin, 2003). Interestingly adrenomedullin immunoreaction products are associated with the outer membranes of mitochondria (Zhou et al., 1999) raising the possibility of adrenomedullin receptors on the mitochondrial membrane with a discrete function on a process that regulates the $\Delta\Psi_m$. Interestingly, where we observed a loss of $\Delta\Psi_m$ in response to KCN plus deoxyglucose, we did not find a similar degree of concomitant release of cytochrome *c* from the mitochondria. Cytochrome *c* release can precede and cause $\Delta\Psi_m$ reduction via caspase activation (Di Lisa et al., 1995), however, there are also data indicating that $\Delta\Psi_m$ reduction is a causative factor leading to cytochrome *c* release (Yoshino et al., 2001). Thus KCN plus deoxyglucose or antimycin can initiate loss of $\Delta\Psi_m$ upstream of cytochrome *c* release and it is at this step where adrenomedullin may be operative to maintain $\Delta\Psi_m$.

There are several important considerations relevant to the study methodology and its extrapolation. The mode of cell death, apoptosis versus necrosis was not examined. This was by design as we sought to focus on total cell death i.e. from apoptosis, necrosis and autophagy and not presuppose a type which would be inherently altered by adrenomedullin. In addition the different types of cell death do overlap or merge together as the degree of ATP depletion can shuttle the predominant form of cell death from apoptosis to necrosis (Prabhakaran et al., 2002). Furthermore, myocytes die by multiple mechanisms and modes of cell death in human heart (Kostin et al., 2003) so a global assessment of cell death is preferable to determine the overall effect on cell viability. The method of assessing cell death, the MTT assay, is dependent on mitochondrial function as a measure of cell death and our method of cell death induction specifically inhibited mitochondrial function so that it is possible that we over estimated cell death and by having cells with insufficient mitochondrial metabolism in still viable cells. However, we have repeatedly used this assay and found that it correlates extremely well with other measures of cell death from trypan blue exclusion to FACS analysis of apoptosis and necrosis (Kong et al., 2005; Kong and Rabkin, 2000; Rabkin and Kong, 2000). Another important consideration is that adrenomedullin plus omapatrilat did not completely reverse cell death and under some conditions

the effects on cardiomyocyte viability appeared to be modest. However, one should not anticipate a complete antagonism by adrenomedullin on the induction of cell death as we selected a very specific mode of cell death induction that should be prevented only by an agent that acts specifically at the site that is interrupted. Similarly, one should not expect to see a complete prevention of the effect of KCN plus deoxyglucose on mitochondrial loss of cytochrome *c*. The smallest impact of adrenomedullin on cardiomyocyte viability was associated with treatment that produced the greatest amount of cell death suggesting that cell death may have been irreversible. The effect of adrenomedullin plus omapatrilat was greater when the amount of cell death was less. For example, adrenomedullin and omapatrilat reduced by about one-half the amount of cell death from antimycin, that alone induced 45% cell death, while the same concentrations of adrenomedullin plus omapatrilat produced only a 16% reduction in cell death in cardiomyocytes that were treated with the combination of antimycin A plus deoxyglucose that produced almost 90% cell death. Perhaps conditions producing milder degrees of cell death may be even more likely to be 'rescued' by adrenomedullin plus omapatrilat. The ability of adrenomedullin to produce a greater prevention of the loss of mitochondrial potential than cytochrome *c* loss, from the mitochondria into the cytosol, appears to be paradoxical. Changes in outer mitochondrial membrane permeabilization, however, coordinates the depolarization of membrane potentials during cell death and can trigger a caspase-independent, presumably cytochrome *c*-independent apoptosis (Dussmann et al., 2003).

5. Conclusion

In summary, the vasodilator peptide adrenomedullin protects cardiomyocytes from cell death induced by interruption of mitochondrial metabolism and inhibition of mitochondrial electron transport. The endopeptidase inhibitor omapatrilat accentuates adrenomedullin-induced cardioprotection. Omapatrilat plus adrenomedullin limited the decline in mitochondrial membrane potential that accompanies interruption of mitochondrial metabolism and reduced cytochrome *c* loss from the mitochondria into the cytosol. An action of adrenomedullin on mitochondria may account for the effect of adrenomedullin to mitigate this mode of cardiomyocyte cell death.

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