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Regulation of Cardiomyocyte Cell Death in Culture

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Introduction

Heart failure is the inability of body's cardiac output to keep pace with its demand for supplies and waste removal. It is characterized by systolic and diastolic ventricular dysfunction, cardiac remodeling, myocyte loss and fibrosis (Nyquist-Battie *et al*, 1996). It is accompanied by an increase in sympathetic activity with measurable increases in circulating plasma norepinephrine levels (Hasegawa et al. 2001; Kaye et al., 1995).

Previous studies have suggested that adrenergic agonists play a role in myocardial cell death. Mann et al. (1992) have shown that increased activation of the adrenergic signaling pathway causes necrotic as well as apoptotic death in cardiomyocytes. It is the goal of this study to determine by which adrenergic receptor-dependent pathway cell death is induced-α1, β1, or β2. After determining which pathway(s) are involved, this study will attempt to clarify by which mechanism myocyte death occurs-necrosis or apoptosis. It is our hope that the data we provide might have some value for future therapeutic treatments of heart failure. Three types of adrenergic receptors are found on cardiomyocytes: α1, β1, and β2. To examine each pathway, three adrenergic agonists were chosen: norepinephrine, isoproterenol, and phenylephrine. Norepinephrine was chosen because it stimulates α1 and β1 adrenergic receptors, with a high affinity for β1. Isoproterenol was chosen because it stimulates β1 and β2 adrenergic receptors, with a high affinity for β2. Phenylephrine was chosen because of its stimulatory nature on a1 receptors.

The cell model used for this study was a clonal muscle cell line derived from embryonic BDIX rat cardiac tissue, given the name H9c2. There are several reasons why this cell line was chosen. First, H9c2 cells express α1, β1, and β2 adrenergic receptors (Zaugg et al. 2000). These cells also display features of hormonal signaling pathways found in adult cardiomyocytes (Dangel et al. 1996). They are easily transfected to manipulate specific protein expression (Heads et al., 1994 and 1995) and have been previously shown to be responsive to oxidative damage in vitro (Aikawa et al., 1997; Hara et al. 1999). Finally, they are easily grown in culture compared to the inherent variability and low yield characteristic of primary culture.

Methods

Cell Culture

H9c2 cells were obtained from the American Type Culture Collection (Rockville, MD, USA). The cells were cultured in Dulbecco's modified Eagle's Medium with 10% fetal bovine serum and 1% penicillin/streptomyocin at 37°C in a humidified 95% air-5% CO_2 atmosphere. Complete medium was changed every 2-3 days. Cells were split by trypsanization into 6-well plates when 80-90% confluence was reached.

Quantification of Cell Death and Cell Lysis

Cell death and lysis was determined using an LDH cytotoxicity kit (Boehringer Mannheim). The LDH assay is a colormetric assay based on the measurement of lactate dehydrogenase (LDH) activity released from the cytosol of damaged cells into the supernatant. The LDH activity is determined in an enzymatic test. In the first step, NAD+ is reduced to NADH/H+ by the oxidation of lactate to pyruvate. In the second enzymatic reaction, 2 H are transferred from NADH and H + to the yellow tetrazolium salt INT to form a red formazan salt. An increase in the

amount of dead or damaged cells results in an increase of LDH enzyme activity in the culture supernatant. This increase correlates with the amount of formazan formed. Thus the amount of color formed in the assay is proportional to the number of lysed cells.

The culture medium was removed from each well after 4 and 24 hours of exposure to the various adrenergic agonists. A 1:1 ratio of culture medium and reaction mixture was created and the solution was incubated in the dark for 15 minutes. HCl was added to stop the reaction and the absorbance was measured at 490nm. To measure intracellular LDH cells were scraped and the procedure was repeated. The amount of extracellular LDH released from the cells was expressed as a percentage of the total amount of LDH.

DNA Fragmentation Detection

A KLENOW Frag-EL DNA Fragmentation Detection kit was used to determine apoptosis. During apoptosis, endogenous endonucleases cleave DNA into oligonucleosomes, generating free 3'OH ends. Klenow binds to the ends and catalyzes the addition of biotin-labeled deoxynucleotides. Biotinylated nucleotides are detected using a streptavidinhorseradish (HRP) conjugate. Diaminobenzidine (DAB) is then added and reacts with the labeled sample to generate an insoluble brown substrate (chromagen) at the site of DNA fragmentation. Light microscopy, morphological changes and chromagen detection can then be used to confirm apoptosis.

Cells were treated with 75ml of 20mg/ml proteinase K (incubated at 22° C-5 min.). To inactivate endogenous peroxidases, cells were treated with 100ml of 3% H_20_2 (incubated at 22° C-5 min.). The cells were then equilibrated with assay buffer and labeled with 60ml of assay labeling reaction mixture. The labeling reaction was terminated with 100ml of blocking buffer for 10 minutes. Blocking buffer was removed and 100ml of conjugate was added (incubated at 22° C-5 min.). 100ml of DAB solution was added (incubated at 22° C-5 min.). Finally the cells were rinsed with deionized water and counterstained with methyl green.

Apoptosis Detection

In order to detect and quantify apoptosis, cells were exposed to a Caspase 3 inhibitor (III). Caspase 3 is a protein cleaving enzyme essential in the disassembly of cells that is activated during apoptosis. It is activated into its active form via a proteolytic cascade of events by other "upstream" caspases in the apoptosis pathway. Its expression virtually guarantees that apoptosis will occur, therefore, its inhibition should prevent cell death.

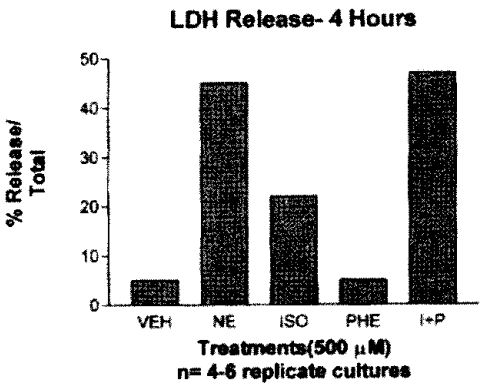
Statistical Methods

All data is expressed as mean ± S.E. A one-way analysis of variance was determined followed by a Tukey-Kramer multiple comparisons test. A value of $p<0.05$ was deemed statistically significant.

Results

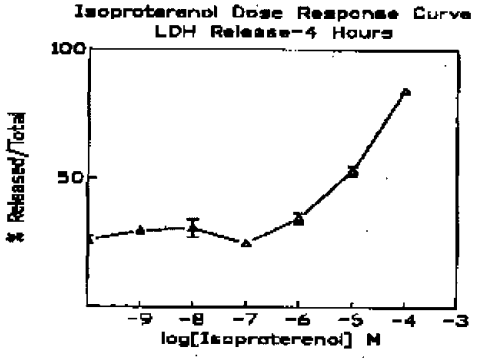
To investigate the effect of adrenergic receptor stimulation on myocyte death, LDH release was measured with agonist-free cells, 500μM norepinephrine (NE) cells, 500μM isoproterenol (Iso) cells, and 500mM phenylephrine (PE) cells after 4 hours of exposure (Figure 1). Results indicate that NE and Iso had approximately the same effect on %LDH release (45.15% and 47.06% respectively, $p<0.001$) compared with the control (5.16%). The PE treated cells showed no effect compared with the control (4.82% vs. 5.16% respectively, $p>0.05$). The combination of Iso and PE demonstrated the same effect as the NE alone (46.18% vs. 47.06% respectively).

Figure 1. Percent LDH release of H9c2 cells after 4 hours of exposure.



Dose response curves were generated for the two agonists that demonstrated cellular membrane damage and cell death, evident by the %LDH release (Figures 2 and 3). The NE dose response curve indicated that a threshold was maintained until concentration levels reached 5μM, after which, a dramatic linear increase in the amount of LDH released corresponded with an increase in concentration up to 500μM (29.85% - 68.68%, $p<0.001$, Figure 2).

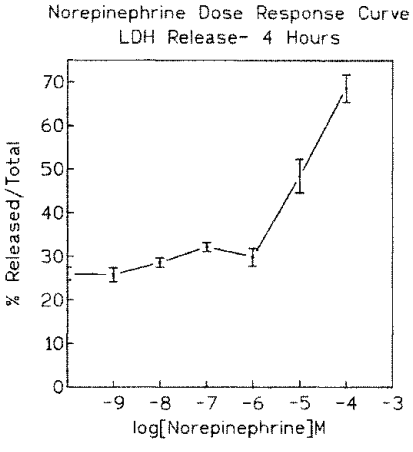
Figure 2. Percent LDH release after 4 hours of exposure to NE at various concentrations.



The Iso dose response curve demonstrated a similar pattern. A threshold was maintained, but unlike the NE curve, the threshold for Iso was slightly lower at 500ηM (Figure 3). Like the NE curve, the Iso curve demonstrated a dramatic linear increase in %LDH release as the concentration increased to 500μM.

To determine whether cell death occurred by necrosis or apoptosis, cells were exposed to a Caspase 3 inhibitor along with 50μM NE and 50μM Iso for 24 hours. Data indicated that cells treated with NE and inhibitor produced a significant decrease in the %LDH released compared to cells treated with NE alone (59.6% vs. 88.1% respectively, $p<0.001$). Data also indicated that cells treated with Iso and inhibitor had no effect compared with Iso alone (83.2% vs. 84.2% respectively, $p>0.05$).

Figure 3. Percent LDH release after 4 hours of exposure to Iso at various concentrations.



The KLENOW Frag-El assay was unable to produce any statistically significant results. The procedure required the cells to remain adhered during the entire procedure. The cells used in this study became dislodged due to rough handling during the procedure. The kit did, however, enable the observation of morphological changes using light microscopy techniques. The cells treated with NE showed hypertrophy,

blebbing, and movement of the chromatin to the nuclear membrane (Figure 4). The cells treated with Iso showed massive rupture of the cell membranes (Figure 5).

Figure 4. Cells treated with NE and Caspase 3 inhibitor.

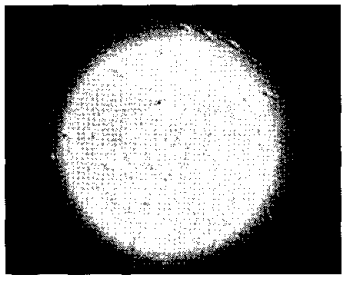
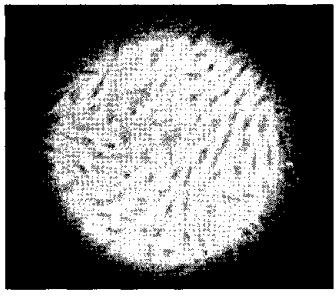


Figure 5. Cells treated with Iso and Caspase inhibitor.



Discussion

Circulating norepinephrine levels are reliable predictors of the severity and outcome of heart failure. Therefore, we hypothesized a neurohormonal influence on the progression of heart failure. Communal et al. (1999) suggested that β1 adrenergic receptor stimulation induces apoptosis and Zaugg et al. (2000) suggested that b-adrenergic apoptotic cell death is largely dissociated from β2 adrenergic receptors and selectively mediated by β1 adrenergic receptors. The results of this study demonstrated that myocyte cell death occurs via a β1 and β2 adrenergic receptor signaling pathway, as evident by the %LDH release compared with the control (Figure 1). However, it is evident by the data that β1 stimulation has a stronger deleterious effect on cell death compared with β2 adrenergic receptor stimulation. There are several reasons why this difference may have occurred. First, the number of β2 adrenergic receptors found on the cell membrane of cardiomyocytes is significantly lower than the number of β1 receptors-approximately a 30:70 ratio respectively. This difference could contribute to the overall effect of sympathetic stimulation that occurs. Second, Communal et al. (1999) suggested that β2 adrenergic receptor stimulation may, in fact, have an inhibiting effect in apoptosis of cardiomyocytes.

To rule out the possibility that NE elicits its effects partly via stimulation of α1 adrenergic receptors, a comparison was done using phenylephrine. The phenylephrine treated cells showed no difference in cell death compared with the control, therefore we concluded that α1 adrenergic receptors are not involved in cell death.

To answer the question of whether the cell death we observed occurred by a necrotic or apoptotic mechanism we treated cells with NE or Iso along with Caspase 3 inhibitor. Because Caspase 3 has a significant role in apoptosis, it was assumed that inhibiting Caspase 3 would inhibit cell apoptotic death. As the data suggest, NE induced cell death appears to occur via an apoptotic mechanism. %LDH was significantly reduced upon exposure of NE treated cells with inhibitor. It was also evident through light microscopy that the cells proliferated and thrived with inhibitor under conditions that normally produce massive cell loss (NE concentration of 50μM). Light microscopy revealed morphological changes characteristic of apoptosis (blebbing, movement of chromatin to nuclear membrane, cell shrinkage). These data suggest that norepinephrine induces myocyte cell death by an apoptotic mechanism via β1 adrenergic receptor stimulation. As for the

effect of the inhibitor on Iso treated cells, no effect was observed. %LDH release was similar to cells without inhibitor, suggesting that the Iso treated cells do not die by apoptosis. Light microscopy revealed that massive cell death took place, with evident rupture of cell membranes characteristic of necrosis. This suggests that isoproterenol induces myocyte cell death by a necrotic mechanism via β2 adrenergic receptor stimulation. In summary, it appears that β1 adrenergic receptor stimulation causes apoptotic death, while β2 adrenergic receptor stimulation causes necrotic death.

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