Protective Effect of Placenta Growth Factor (PlGF) Against Hypoxia-Reoxygenation and Serum-Deprivation Induced Apoptosis in Neonatal Rat Cardiomyocytes

Kyle Bohman[1], Marin Schweizer[1], Bob Berendt[1], Donald S. Torry[2], Ronald J. Torry[1]
[1]Pharmaceutical Sciences, Drake University, Des Moines, IA; [2]School of Medicine, Southern Illinois, Springfield, IL

ABSTRACT

Placental Growth Factor (PlGF) has been shown to play a significant role during pathological events such as ischemia. PlGF knockout mice developed normally, but had significantly impaired angiogenic reactions to ischemia and tumor formation (Falco et al 2002). The role of PlGF will be investigated during apoptosis induced by serum-deprivation and hypoxia-reoxygenation--physiological conditions relevant to ischemic cardiomyopathy. Likewise, serum-deprivation occurs during ischemic conditions common in cardiomyopathy and triggers apoptosis through the mitochondrial pathway (Bialik et al 1999). Hypoxia-reoxygenation mimics ischemia-reperfusion, and causes apoptosis by producing intolerable levels of reactive oxygen species.

We intend to study the effect of varying PlGF treatments on caspase –dependent apoptosis in neonatal rat cardiomyocytes during serum-deprivation and hypoxia-reoxygenation.
Supported by R15-HL72802-01 (RJT)

BACKGROUND

Myocardial ischemia/infarction is a leading cause of morbidity and mortality in the United States. Therapies to enhance coronary angiogenesis and/or reduce myocyte apoptosis could substantially reduce morbidity/mortality following cardiac ischemia. However, the endogenous mechanisms for these protections are not well known. One hypothesis would involve the production of specific factors from cardiomyocytes that then act to induce the growth of the surrounding vasculature and/or reduce apoptotic cell death.

There is considerable evidence that members of the vascular endothelial growth factor (VEGF) family play an important role in these events. One VEGF family member that has received relatively little attention in terms of its expression and function in heart tissue is placenta growth factor (PlGF). PlGF has been shown to act synergistically with vascular endothelial growth factor (VEGF) to promote angiogenesis and inhibit apoptosis. We and others have shown significant upregulation of VEGF expression in models of coronary angiogenesis (Torry et al., 2001). Furthermore, we have demonstrated that PlGF mRNA expression is increased in response to cardiomyocyte hypoxia (Stalheim, 2003). Others have shown PlGF knockout mice exhibit reduced coronary angiogenesis following myocardial infarction and increased endothelial cell apoptosis (Carmeliet et al., 2001). It is not known if PlGF can protect cardiomyocytes from apoptosis.

The present study sought to determine if PlGF treatment affects the amount of caspase-dependent apoptosis in cardiomyocytes during: 1) serum-deprivation and 2) hypoxia-reoxygenation.

METHODS

Rat neonatal cardiomyocyte cell culture:

Rat neonatal cardiomyocytes were isolated and cultured as described (Zheng et al., 2001). Purity of cultures were assessed by indirect immunocytochemistry using monoclonal antibody against sarcomeric myosin (MF-20; Univ Iowa Hybridoma Center) and propidium iodine counterstain.

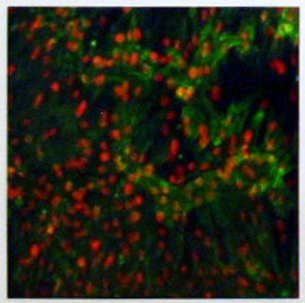
Culture Treatments:

Hypoxic conditions were established by placing cell cultures in an anaerobic pouch (BBL), and reoxygenated by removing the cultures from the anaeroabic pouch. Serum-Deprivation was applied by replacing normal 10%FBS DMEM media with DMEM media lacking FBS. The positive apoptosis control was 1uM Staurosporine (Sigma). The negative apoptosis control was 20mM Z-VADfmk (Promega), a pan-caspase inhibitor.

Apoptosis detection:

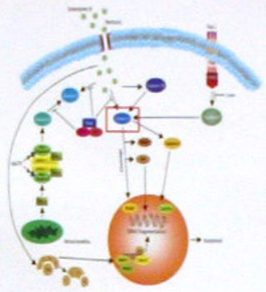
Caspase-3,7 luminescence assay (Promega) was used to determine the level of activated Caspases 3 and 7 in the cells of each well of cultured rat neonatal cardiomyocytes. The values of treated wells were compared to untreated ("normoxic") wells to calculate relative luminescence values. Protein concentrations of each well were determined using a Lowry protein assay (BioRad). Caspase luminescence values were normalized based on protein concentration of each well to produce more accurate relative Caspase values.

Cultured Neonatal Rat Cardiomyocytes



Cardiac cells were isolated from 2-3 day old rats, selectively plated to enrich cardiomyocytes or non-cardiomyocytes. Representative aliquots were collected and cultured on chamber slides in DMEM/10% FBS for 48hrs. The cultures were then washed, fixed with -20°C acetone, re-hydrated in PBS and reacted with monoclonal antibody to sarcomeric myosin (UI Hybridoma Center) and then FITC-conjugated goat anti-mouse IgG antibody (Dakopatts). The cells were counterstained with propidium iodine to label all nuclei. Cultures labeled as cardiomyocytes contained >85% sarcomeric myosin positive cells.

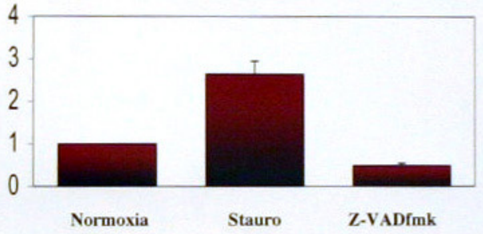
Caspase and Apoptosis



Caspases are a series of cysteine aspases normally present in cells in the inactive Procaspase form. Upstream signals from both the extrinsic and intrinsic apoptotic pathways activate the caspases. Caspases 3 and 7 are at the end of the Caspase chain and necessarily lead to apoptosis. They are the key mediators of apoptosis and act on a number of substrates to cause apoptotic death.

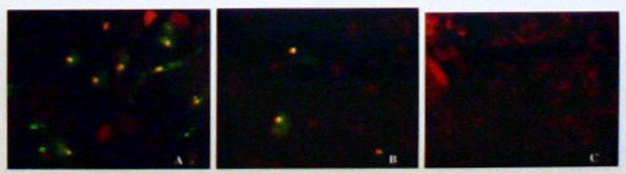
RESULTS

Fold Caspase-3,7 Activity



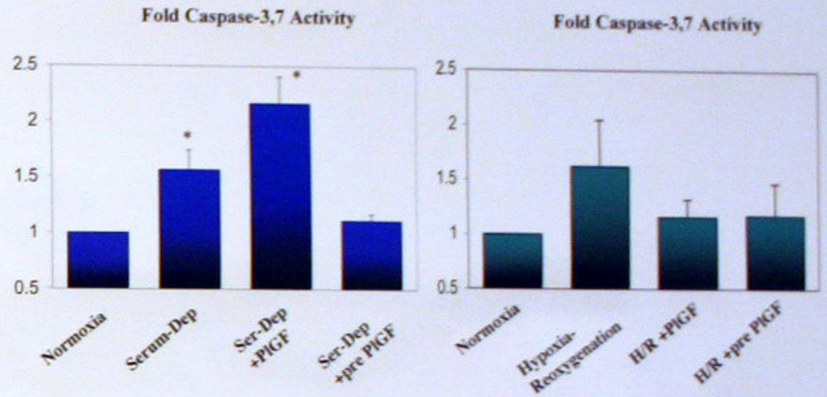
Cardiomyocytes were cultured under normoxic conditions which modeled, as accurately as possible, physiological conditions. Positive control cardiomyocytes were treated with Staurosporine (1uM) to induce the maximum level of caspase activity. Negative control cardiomyocytes were treated with Z-VADfmk (20mM), a pan-caspase inhibitor.

In-Situ FITC Labeling of Caspase 3



Neonatal rat cardiomyocytes were cultured and treated to induce apoptosis. A FITC-conjugated Z-VADfmk in situ marker (Promega) was used to specifically label active caspases. A) H/R with 1%FCS; B) H/R with 10%FCS; C) Normoxia with 1%FCS. Active caspase 3 is found in the cytosol. The FITC labeled active caspase can be visualized above.

Caspase 3,7 Activity Induced by Serum-Deprivation and Hypoxia-Reoxygenation and the Effect of PlGF Treatment



Cardiomyocytes were isolated from neonatal rats and cultured in DMEM/10% FBS. Two days after culturing, treatments were applied. Cardiomyoctyes were deprived of serum and treated with PlGF, then compared to normoxic cultures (A). Cardiomyocytes were placed in hypoxic conditions for 36 hours followed by 6 hours of reoxygenation in normoxic conditions (B). Relative Capase-3,7 Activity was quantified with Caspase-GLO assay (Promega). Kruskal-Wallis non-parametric statistical test was used to evaluate the significance of the results. The test revealed statistical significance for the Serum-Deprivation treatment group ($p=0.0033$). The test did not reveal statistical significance for the Hypoxia-Reoxygenation treatment group.

SUMMARY AND CONCLUSION

Effect of Placenta Growth Factor:

1. 8-12 hour pretreatment of PlGF (25ng/ml) significantly decreased apoptosis induced by Serum-Deprivation. Serum-Deprivation caused apoptosis levels to rise to an average of 1.564 times normoxic levels and an 8-12 hour pretreatment with PlGF before Serum-Deprivation reduced apoptosis to an average level of 1.114 times normoxic levels.
2. PlGF (25ng/ml) and 8-12 hour pretreatment of PlGF (25ng/ml) decreased apoptosis induced by Hypoxia-Reoxygenation although the results were not statistically significant. Hypoxia-Reoxygenation caused an increase of apoptosis to an average level of 1.621 times normoxic levels. Treatment with PlGF and an 8 hour pretreatment with PlGF reduced the level of apoptosis on average to 1.159 and 1.175 times normoxic levels, respectively.

Conclusion:

The results of the study showed that a pretreatment of Placenta Growth Factor protects neonatal cardiomyocytes from apoptosis induced by Serum-Deprivation. The results also demonstrated that PlGF tends to protect neonatal cardiomyocytes from apoptosis induced by Hypoxia-Reoxygention, but the data failed to reach significance due to variability. It is suspected that a pretreatment with PlGF activates a slow or delayed molecular cascade or leads to the upregulation of one or many anti-apoptotic genes to protect cardiomyocytes against apoptosis.

Supported by R15-HL72802-01 (RJT)