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# Erythropoietin Regulation on p38 MAPK and Downstream Targets of AKT during Hypoxia/ Reperfusion Injury on H9C2 Cells.

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**ABSTRACT**

Acute myocardial infarction is a major cause of premature mortality in developed countries and is largely associated with Ischemia/Reperfusion (I/R) injury. Erythropoietin (EPO) is a hematopoietic cytokine, and its receptor is shown to be present in tissues outside blood, including the heart. EPO is strongly inferred to protect the cardiomyocytes from the reperfusion injury and our aim is to elucidate the mechanistic pathway behind the cardioprotective effect of EPO. H9C2 cells were exposed to H/R (Hypoxia/Reperfusion) with or without pretreatment using 10, 15 and 20 U/ml of EPO. The Phosphorylation of p38 MAPK, BAD, XIAP, Cytochrome - c were analyzed by Western blot. Caspase-3 activity was determined by Caspase-3 colorimetric assay. EPO increased the phosphorylation of p38 MAPK, Akt, BAD and XIAP compared to HR. EPO prevented the caspase-3 activations induced by HR. Further H9C2 cells blocked with Wortmannin and SB203580 showed increase caspase-3 activity and thus abolishes the protective effect of EPO. We have confirmed that EPO increase phosphorylation of p38 MAPK and prevent H9C2 from H/R induced cell death. Together these findings support mechanistic evidence for the protective effect of EPO in cardiomyocytes to prevent H/R induced cell death and possibly create new avenues for effective cardioprotective therapeutics.

**Keywords:** H9C2 cells, Erythropoietin, Hypoxia/Reperfusion injury, p38 MAPK, XIAP

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## INTRODUCTION

Erythropoietin (EPO) is a glycoprotein hormone/cytokine that is produced primarily in the peritubular interstitial cells of the kidney [1] with a molecular weight of 30.4 kDa. EPO secret from the kidney in response to hypoxia, to maintain normal erythropoiesis and oxygen levels in healthy adults. In 1987, recombinant human EPO (rhEPO) was manufactured and used to treat anemia of chronic renal failure. It has been found that the EPO also possess cardio protective function which is mediated by EPO-R and its signaling pathways [2]. At a dose of 10,000 U/kg/d, EPO showed protection against cardiac remodeling after MI in *in-vivo* mouse models [3]. In neonatal mouse, cardiomyocytes showed 50% of protection from apoptosis induced by 1µM of doxorubicin when EPO given at a dose of 1 U/ml. EPO protects apoptosis in neonatal rat heart ventricular myocytes by activation of PI3K/Akt when exposed to hypoxia [4]. The cardioprotective action of EPO against H/R injury is mediated by PI3K/Akt and ERK1/2 pathways which are believed to be reperfusion-injury salvage kinase pathway and is also associated with reduced caspase-3 activation. Blocking of these salvage kinase pathway by LY294002, or U0126 (PI3K inhibitor), the ERK1/2 inhibitor, showed a loss in the cardioprotective effect of EPO [5]. A recent clinical study has shown a strong evidence of EPO's anti-necrotic effect of bisphosphonate-associated osteo-necrosis and radio osteo-necrosis of the jaw in cancer patients [6].

Previously we have shown that EPO protection against apoptosis, necrosis, $\Delta\psi_m$ , $Ca^{2+}$ homeostasis though Akt pathway during H/R injury [7]. We still want to find the EPO's action on p38 MAPK pathway and downstream targets of Akt in H9C2 cells during H/R. We found out that multiple downstream effectors of Akt pathway, including phosphorylation of BAD, XIAP and p38 MAPK, decreased activity of caspases 3 and cytochrome c release into the cytosol.

## MATERIAL AND METHODS

### Cell culture and Treatment

H9C2 cells were cultured to 70–80% confluency and then serum starved in basal medium (DMEM + 0.1% Bovine Serum Albumin) for 24 hrs. H9C2 cells received no intervention (normoxic controls) or were exposed to H/R after pretreatment with two applications of EPO [10 U/ml, 15 U/ml and 20 U/ml). Treatment of EPO was accomplished 24 hrs before (first application) hypoxia. Second application during the induction of hypoxia and this hypoxic conditions were obtained by an incubation at 37°C in a small airtight chamber containing H9C2 cells with 94% $N_2$, 5% $CO_2$ remaining 1% $O_2$ for 8 hrs in the presence of serum and glucose free DMEM medium [19]. Hypoxic medium were discarded and cell were reperfused with glucose containing DMEM + 10% FBS for 16 hrs. For some of the experiments, cells were treated with 10 µM SB203580 (SB), p38 MAPK inhibitor or 1 µM Wortmannin (WT), PI3K/AKT inhibitor before 30 mins of each application of EPO. Throughout the experiments control cells were maintained in DMEM+10% FBS [7].

### Western Blot analysis

H9C2 cells were cultured in 60 mm dishes to 80% confluency and were treated under normoxic conditions or 8 hrs hypoxia and 30 mins reperfusion with or without EPO and 1 mM Wortmannin before 30 mins of each application of EPO. Cells were kept on ice and washed thrice with ice cold PBS. Proteins were solubilized and extracted with 100 ml RIPA buffer (50 mM Tris pH 8.0, 150 mM NaCl, 0.5%SDS, 1% Nonidet P40, 0.5% sodium deoxycholate, 1 mM EDTA, 1X protease and phosphatase inhibitor cocktail (Cell Signaling technologies). The lysate were used to estimate protein content with the Bradford Assay Reagent. Equal amounts of protein (10–60 mg) from each sample were electrophoresed on a 10% SDS-polyacrylamide gel with running buffer and transferred to nitrocellulose membrane. The membranes were treated with primary antibody for p 38 MAPK, pp 38 MAPK, BAD, p BAD, XIAP and cytochrome c (Cell Signaling Technologies) at a dilution of 1:1000, for overnight incubation at $4^0$ C. They were again washed 3 times before incubating with matched secondary antibody (1:5000) for 45 mins. The protein bands were developed with Alkaline Phosphatase substrate [7].

### Caspase-3 colorimetric assays

Cells were cultured in 60 mm dishes to 70–80% confluency, and subjected to H/R or normoxia with or without pre-treatment with EPO as described earlier. At the end of treatment the cells were collected by

centrifugation, washed twice with ice-cold PBS and resuspended in lysis buffer as given in the caspase-3 colorimetric Assay kit (BF3100) R & D systems. The cell lysate was incubated on ice for 10 mins and centrifuged at 10,000-x g for1 mins. The supernatant was transferred to a new tube and kept on ice. In 96-well plates, 50 ml (2 mg/ml) of cell lysate were added and 50 ml of 2X Reaction Buffer with 10 ml of fresh DTT stock per 1 ml of 2 X Reaction Buffer. Then 5ml of caspase-3 colorimetric substrate (DEVD-pNA) were added to each reaction well and incubated at 37uC for 1–2 hrs and measured the readings on a microplate reader at a wavelength of 405 nm [7].

**Statistical Analysis**

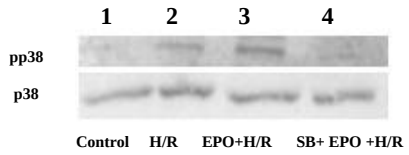
Numerical data were presented as means ± SEM from three samples in all the experiments. The difference between groups was analyzed using ANOVA followed by TUKEYs test when permitted. Values for P< 0.05 were considered as statistically significant.

## RESULTS

**EPO Increases the Phosphorylation of p38 MAPK**

Samples pretreated with EPO showed increase in p38 MAPK phosphorylation in lane 3 when compared to control and H/R. The phosphorylation of p38 MAPK was blocked by SB203580 in lane 4. Expression of p38 MAPK (loading control) in the corresponding row is not altered (Fig.1).

**A**




**B**

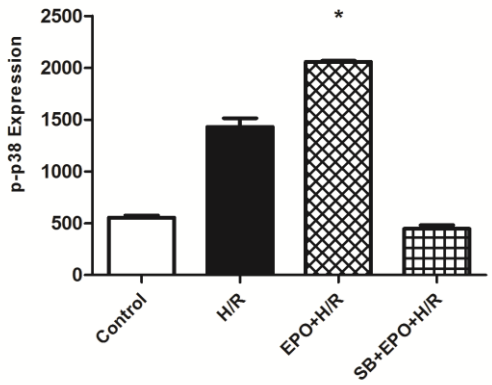



**Figure 1: Depicts the phosphorylation of p38 MAPK in H/R-induced H9C2.**

**EPO Increases the Phosphorylation of Bad**

Samples pretreated with EPO showed an increase in phosphorylation of BAD in lane 3 as compared to samples exposed to H/R without EPO pretreatment in lane 2. Expression of BAD (loading control) in the corresponding row is not altered (Fig.2).

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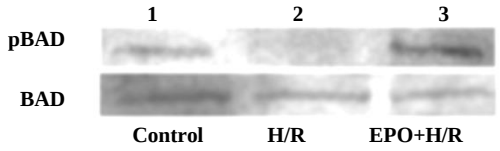
A

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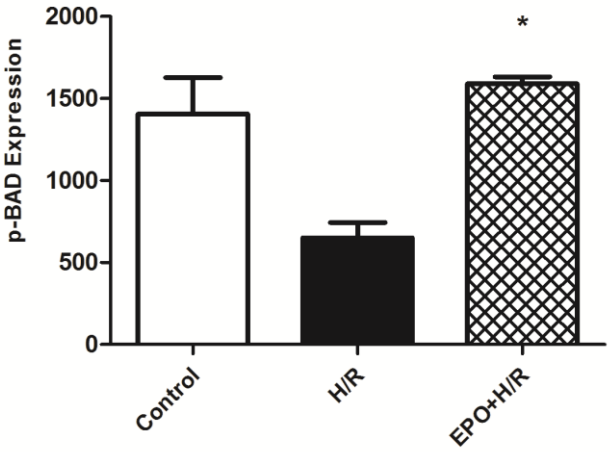
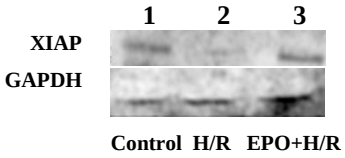



**Figure 2: Depict the phosphorylation of BAD in H/R-induced H9C2**

**EPO Increases the Intracellular Levels of XIAP**

The H9C2 cells pretreated with EPO showed an increase in intracellular levels of XIAP in lane 3 as compared to H9C2 cells exposed to H/R alone in lane 2 (Fig.3).

A




B

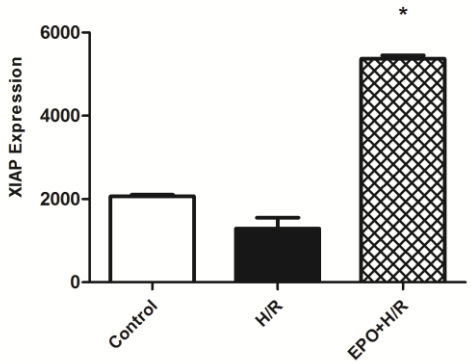



**Figure 3: Depict the effect of EPO on XIAP in H9C2 cells**

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**EPO Decreases Cytosolic Release of Cytochrome C**

EPO pretreated samples showed decrease cytosolic release of cytochrome c (cytosolic cytochrome c lane 3) and increased mitochondrial accumulation of cytochrome c (mitochondrial cytochrome c lane 3) but samples without EPO pretreatment showed increased cytosolic release of cytochrome c (cytosolic cytochrome c panel 2) and decreased mitochondrial accumulation of cytochrome c (mitochondrial cytochrome c lane 2)(Fig.4).

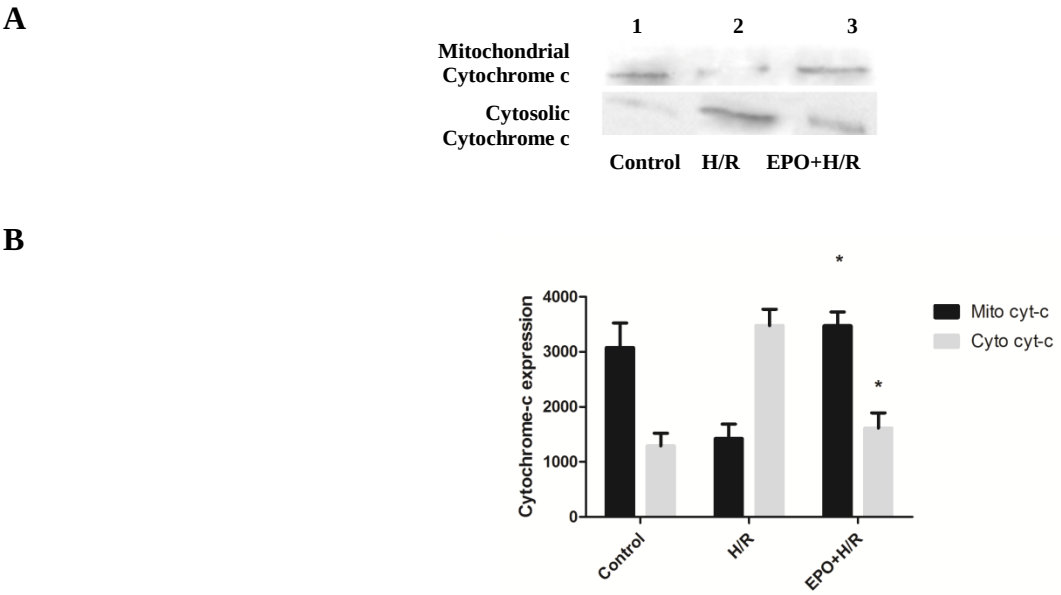


**Figure 4: depicts the cytosolic and mitochondrial release of cytochrome c in H/R-induced H9C2**

**EPO INHIBITS CASPASE-3 ACTIVITY**

H9C2 cells were induced with H/R with or without EPO treatment and the caspase-3 activity was measured. The caspase-3 activities were markedly elevated after H/R. Pretreatment with EPO prevented the caspase-3 proteolytic activations induced by H/R. Further samples blocked with WT and SB203580 showed an increase in caspase-3 activity (Fig. 5).

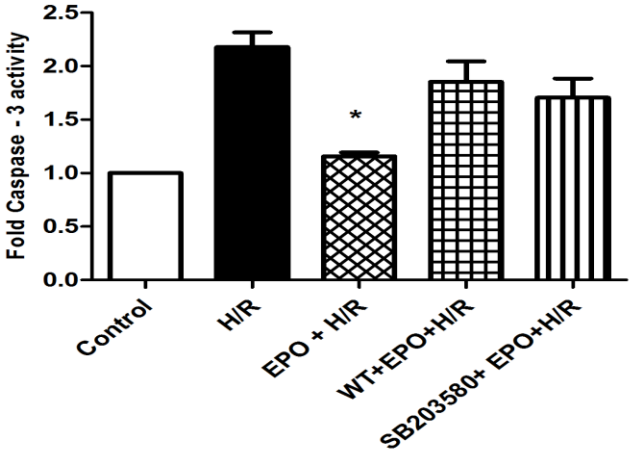


**Figure 5: Caspase -3 activity shows that the protective effect of EPO was abolished when treated with WT or SB**

**DISCUSSION**

Our recent study shows that 20 U/ml EPO pretreatment protects H9C2 cells from H/R injury and the protection is via Akt survival pathway [7]. In this present study we want to figure out the role of p38 MAPK pathway and downstream targets of Akt during H/R injury.

Abundant evidence for p38 involvement in apoptosis exists to date and is based on concomitant activation of p38 and apoptosis induced by a variety of agents such as NGF withdrawal and Fas ligation [8-11]. It must be mentioned that the role of p38 in apoptosis is cell type and stimulus dependent. While p38 signaling has been shown to promote cell death in some cell lines, in different cell lines p38 has been shown to enhance survival, cell growth, and differentiation [11]. EPO protects the cerebral ischemic damage by protein kinase signaling pathway [12]. The same signaling pathways also protect the cardiovascular ischemic damage [13]. We figured out EPO might also confer cardioprotection during H/R injury. The protective effect of EPO is also abolished when blocked with SB203580 (p38 MAPK inhibitor).

In our present study, beneficial effects of EPO were observed in H9C2 cells. The samples pretreated with EPO post H/R showed decreased caspase-3 activity when compared to H/R alone. Control cells also showed decreased caspase-3 activities when compared to H/R alone. The preincubation of Wortmannin for 30 min before EPO and post-H/R showed increase in caspase-3 activity when compared to EPO pretreated samples. Similarly, samples which were preincubated with SB208530 showed increase in caspase-3 activity when compared to EPO pretreated samples.

The samples pretreated with EPO showed decrease cytosolic release of cytochrome c and increased mitochondrial accumulation of cytochrome c in contrast to H9C2 without EPO pretreatment which showed increased cytosolic release of cytochrome c and decreased mitochondrial accumulation of cytochrome c.

Previous studies reported that EPO conferred protection against apoptosis in erythroid progenitor cells via modulation of caspase (caspase 1-, caspase 3-, and caspase 8-like) activity [14]. Effector caspase-1 and caspase-3 cleaves many cellular proteins and precedes the death pathway [15].

Increased phosphorylation of pBAD has seen in EPO pretreated samples post-H/R when compared to H/R alone. One of the downstream targets of Akt is BCL-2-associated death promoter (BAD]. BAD belongs to BCL-2 family and it includes both anti-apoptotic proteins and pro-apoptotic protein. When Akt phosphorylates BAD, it forms the BAD-(14-3-3) protein homodimer and allows BCL-2 which is free to inhibit apoptosis triggered by BAX. BAD phosphorylation is anti-apoptotic, and dephosphorylation is pro-apoptotic [16].

Another downstream target of Akt is XIAP (X-linked inhibitor of apoptosis) [17]. XIAP belongs to the family of baculovirus inhibitor of apoptosis repeats (BIR) that opposes the post-mitochondrial steps in the intrinsic pathway [18]. XIAP binds to and inhibits caspases-9, 3, and 7. It also inhibits procaspase-9 activation in the apoptosome by preventing its dimerization and thus protects the cell from accidental activation of caspases [19]. Hence, inhibitor of apoptosis (IAP) proteins inhibits the initiator caspase, caspase-9 and the executer caspase, caspases-3 and 7. In our present study, pretreatment of EPO increased the expression of both BAD and XIAP in H/R induced H9C2 cells when compared to H/R alone.

## CONCLUSION

EPO prevents the release of cytochrome c into the cytoplasm and reduced the activity of caspase-3. EPO activates multiple signaling pathways that contribute to the observed inhibition of cell death induced by H/R injury p38 MAPK and downstream signaling pathways of Akt such as BAD and XIAP. Whilst this manuscript does not present a whole picture of these mechanisms, our findings support mechanistic evidence for the protective effect of EPO in H9C2 cells to prevent H/R-induced cell death and possibly create new avenues for effective cardioprotective therapeutics.

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