CHAPTER 1

INTRODUCTION

1.1 HEART FAILURE

Heart Failure (HF), is a state in which the heart cannot provide sufficient cardiac output to satisfy the metabolic needs of the body. It is a major health problem worldwide (Gao & Koch 2009). Despite the progress that has been made in the treatment of chronic HF (CHF), the overall morbidity and mortality remains high. It is a syndrome arising from multiple causes that affects one in five adults in their lifetime (Levy et al 2002). It is a common end point for many diseases of the cardiovascular system and is caused by increased workload, restricted filling, and myocyte loss. Therefore, it is imperative and there is a constant need to understand the molecular mechanisms responsible for this disease to discover and develop novel therapies (Levy et al 2002).

Myocardial infarction and hypertension, can lead to an increase in cardiac workload and elevated mechanical stress on cardiomyocytes. But the loss of cardiomyocytes by apoptosis has been mostly associated with myocardial infarction, I/R injury, or end-stage HF (Kim et al 2003). Signaling molecules that transduce the signals from this extracellular stress to different cellular compartments play a central role in mediating the hypertrophic process and leads to the HF (Wang et al 1998). But in many cases hypoxia is not the lone cause for the myocardial infarction or I/R injury, instead it is the consecutive reoxygenation which contributes to the apoptosis of
cardiomyocytes. Hypoxia and reoxygenation are well-known causes of cellular injury and death in a variety of cell types. Over the past several years, it has become clear that hypoxia and reoxygenation can trigger the apoptotic cell death as well as necrosis in a number of cell types (Malhotra et al 2001).

1.1.1 Epidemiology of Heart Failure

HF is a dangerous disease and tackling this problem must be believed as worldwide health precedence. In 2008, about 17.3 million people reported died from CVD (cardiovascular disease). At present, around 26 million people are living with HF globally. It is estimated that in 2030 about 23 million people will die annually. CVD has been a dominant killer disease among the Western countries, but now its prevalence in India is significant. The Associated Chambers of Commerce and Industry of India (ASSOCHAM) published a report that in India the incidence of HF has been increased over last two decades. About 1.3 to 4.6 million people are suffering from CVD, obesity and diabetes. The major factors which lead to HF are consumption of alcohol, smoking, lack of sleep, unhealthy eating habits and most importantly stress. Furthermore, in India more than 50% of heart attack patients die because of time delay to reach hospital. CVD is the leading cause of death in almost all the states of India. Top five diseases that lead to death in various types of populations are shown in the Table 1.1. CVD is the biggest cause of mortality in all these populations (Gupta et al 2012).
Table 1.1 Top 5 reasons of deaths in India classified according to the gender and the areas of residence

<table>
<thead>
<tr>
<th>Rank</th>
<th>India (All age groups)</th>
<th>Economically backward states</th>
<th>Economically advanced states</th>
<th>Rural populations Urban</th>
<th>Urban populations Men</th>
<th>Men</th>
<th>Women</th>
<th>Middle-age (25-69 yr) Cardiology</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Cardiovascular</td>
<td>Cardiovascular</td>
<td>Cardiovascular</td>
<td>Cardiovascular</td>
<td>Cardiovascular</td>
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<td>Cardiovascular</td>
</tr>
<tr>
<td>2</td>
<td>COPD, asthma</td>
<td>Diarrhea</td>
<td>COPD, asthma</td>
<td>Cancers</td>
<td>COPD, asthma</td>
<td></td>
<td></td>
<td>Diarrhea</td>
</tr>
<tr>
<td>3</td>
<td>Diarrhea</td>
<td>Respiratory infections</td>
<td>Cancers</td>
<td>COPD, asthma</td>
<td>Tuberculosis</td>
<td></td>
<td></td>
<td>COPD, asthma</td>
</tr>
<tr>
<td>4</td>
<td>Perinatal</td>
<td>COPD, asthma</td>
<td>Senility</td>
<td>Tuberculosis</td>
<td>Diarrhea</td>
<td></td>
<td></td>
<td>Respiratory infections</td>
</tr>
<tr>
<td>5</td>
<td>Respiratory infections</td>
<td>Perinatal</td>
<td>Diarrhea</td>
<td>Senility</td>
<td>Perinatal</td>
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<td>Senility</td>
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</tbody>
</table>
Urbanization and economic growth has a major hand of this disease in India. People adopting an unhealthy lifestyle with loss of physical activity, large intake of fatty foods and tobacco and increasing stress levels has led to increase in the incidence of this disease. A recent data reported that around 30% of the population living in urban and 15% of the rural population are prone to heart attacks and high blood pressure (Gupta et al 2012).

1.2 HYPOXIA

HF can result from variety of causes and hypoxia is one among them. Hypoxic state of the heart is the most common and dangerous condition that result in insufficient supply of oxygen to the cardiac cells, below their metabolic requirement. The World Health Organization has declared that acute coronary occlusion would be one of the major causes of death globally, by the year 2020. Cells undergo apoptosis during hypoxia, to prevent the buildup of hypoxia-induced mutated cells (Huang et al 2007), presumably because hypoxia induces genetic instability by the induction of fragile sites causing gene amplification (Coquelle et al 1998) and reduces DNA mismatch repair activity.

1.2.1 Hypoxia Mechanism of Action

In the heart, gene expression is adjusted to oxygen availability through the regulation of gene transcription by the hypoxia-inducible basic helix-loop-helix transcription factor hypoxia-inducible factor 1α (HIF-1α) (Huang et al 2004). The transcription of HIF-1α involves an extensive range of genes which plays a major role in erythropoiesis, angiogenesis, inflammation, vascular remodeling, apoptosis, reactive oxygen species (ROS), vasomotor reactivity and vascular tone, in the cytoplasm, the oxygen-dependent degradation (ODD) domain of HIF-1α is modified by a HIF-prolyl hydroxylase under normoxic conditions. Due to this post-translational
modifications, HIF-1α recognition by pVHL (product of the von Hippel Lindau tumor suppressor protein), and consequent degradation occurs by proteasome ubiquination (Ub) occurs. Whereas in the nucleus during normoxia, an asparaginyl (Asn) hydroxylase modifies the C-TAD domain of HIF-1 α and later blocks its binding with the transcriptional coactivator p300. Proteosome destructs the polyubiquitylated HIF-1α and is stabilized during hypoxia, since hydroxylation does not occur (Semenza 2001). Heterodimerization of HIF-1α with aryl hydrocarbon nuclear translocase (ARNT) forms the active HIF complex that binds to a core hypoxia response element and a wide array of genes that plays a protective function in the cardiovascular system (Chi & Karliner 2004) as shown in the Figure 1.1.

![Diagram of HIF-1α gene activation during hypoxia](image)

**Figure 1.1** HIF-1α gene activation during hypoxia
1.3 REACTIVE OXYGEN SPECIES GENERATION MECHANISM

Atmospheric oxygen is a diatomic molecule with the formula $O_2$, in which the two oxygen atoms are double bonded to each other (triplet oxygen). This form of oxygen has two unpaired electrons, defining it as a radical. Whereas most radicals are highly reactive molecules, triplet oxygen is fortunately unreactive. Triplet oxygen is released by plants as a product of photosynthesis and is necessary for aerobic respiration in mammalian cells. In mammalian cells, aerobic respiration is coupled to the generation of ATP, which is required in both cell-type-specific functions and for the maintenance of viability (Chandel & Budinger 2007). Because of its radical nature, oxygen can undergo unintended electron transfer reactions with cellular constituents, with damaging consequences. It is therefore unsurprising that mammalian cells have developed complex mechanisms to recognize fluctuations in environmental oxygen levels. In a variety of systems, ROS have been reported to be produced by the mitochondria, the NAD(P)H oxidase, or other cellular oxidant-generating systems in cells exposed to hypoxia or hyperoxia (Chandel et al 1998). These ROS are required for activation of the signaling pathways induced by these stimuli. The signaling pathways that are activated and their downstream consequences, differ drastically after exposure to hyperoxia or hypoxia. In oxygen dependent ROS generation is prone to forming reactive oxygen species by a stepwise monovalent reduction reaction to form superoxide ($O_2^-$), a reactive anion (Ježek & Hlavatá 2005; Skulachev 2006) as shown in Figure 1.2. Superoxide has an unpaired electron and, relatively unstable when compared with triplet oxygen. A dismutation reaction of superoxide anion with water can result in the formation of hydrogen peroxide and oxygen.
Figure 1.2 Stepwise formation of reactive oxygen species

The mitochondrial electron transport chain can generate superoxide at Complexes I, II, and III (Chandel et al 1998). Complexes I and II generate superoxide into the mitochondrial matrix, whereas Complex III has the ability to generate superoxide into both the mitochondrial intermembrane space and the mitochondrial matrix (Figure 1.3A).
Figure 1.3  The generation of ROS by mitochondrial electron transport chain (A), the Q cycle (B)

Complex III produces superoxide from the radical ubisemiquinone during the Q cycle. Ubisemiquinone, which generates superoxide, is generated at two distinct sites, the Qi and Qo sites, which release the
superoxide into the matrix and the intermembrane space, respectively (Figure 1.3). ROS do not seem to be generated by Complex IV (Turrens 2003).

The other major source of superoxide generation in the cell is the plasma membrane NAD(P)H oxidase (Irani 2000). The NAD (P) H oxidase is a multiprotein complex formed by the assembly of resident membrane proteins, including gp91phox, p22phox and by the recruitment of cytosolic proteins, including p47phox, p67phox, p40phox, and the small GTPases Rac1 and Rac2 (Griendling et al 2000). Superoxide production by NAD(P)H oxidase is required for the neutrophil oxidant burst. In addition, the assembly and generation of ROS by the NAD(P)H oxidase has shown to be required for the production of cytokines in alveolar macrophages in a variety of disease models as shown in the figure 1.4 (Le Bras et al 2005).

![Diagram of ROS generation](image)

**Figure 1.4** The generation of ROS in the cell by NAD(P)H oxidase system and mitochondrial electron transport
1.4 ISCHEMIA

Ischemia refers to the insufficient blood flow to a tissue, usually the heart. This is one of the main causes of hypoxia, and this type of hypoxia is termed ischemic hypoxia. This can occur due to a gradual blockage in the coronary artery. Usually ischemia is a temporary condition. The blood supply may be normal during the resting state, but any kind of exertion or exercise may result in insufficient blood flow to the heart muscles. Chronic cases of Ischemia may arise due to the gradual narrowing of the arteries, which may result in limited blood flow even when at rest, and this may result in a gradual weakening of the heart.

![Heart Attack diagram](image)

**Figure 1.5** Ischemic heart with cholesterol plaques in the artery supplying blood to the heart
The mammalian heart is an obligate aerobic organ. The heart consumes approximately 8 - 15 ml O\textsubscript{2}/min/100 g tissue when compared to brain consumption (approximately 3 ml O\textsubscript{2}/min/100 g tissue) (Giordano 2005; Henning & Olsson 2006). Mammalian heart muscle cannot produce enough energy to maintain essential cellular processes under anaerobic conditions; a constant supply of oxygen is indispensable to sustain cardiac function and viability. A major determinant of myocardial gene expression is oxygen and the expression patterns of gene in the heart are significantly altered during isolated hypoxia or ischemia-associated hypoxia. The myocardium can tolerate brief periods (up to 15 minutes) of severe and even total myocardial ischemia without consequential cardiomyocyte damage. Although the cardiomyocytes suffer an ischemic injury, the damage caused by ischemia can be reversible with prompt arterial reperfusion (Huang et al 2004).

1.4.1 Reperfusion

Reperfusion is the process of re-introducing oxygen to the deprived cells. This process is therapeutic for most cases resulting in hypoxia, and is necessary to resuscitate the ischemic myocardium. Timely reperfusion aids cardiomyocyte salvage and decreases cardiac morbidity and mortality. However, the occurrence of reperfusion can also result in tissue damage, and this is called reperfusion injury, a common occurrence in ischemic tissues.

1.4.2 Ischemia-reperfusion (I/R) injury

In mitochondria ATP is generated; it consumes huge quantity of oxygen during regular perfusion and maintains a normal ROS generation for scavenging action. It plays a role in stabilization of calcium homeostasis and cellular ion homeostasis. In contrast, during ischemia due to the lack of oxygen supply the electron flow gets inhibited and ATP utilization turns out
to be inefficient. The main function of proton-translocating F$_0$F$_1$ ATP synthase is to produce ATP but during ischemia it has become an F$_0$F$_1$ ATPase which consumes ATP and efflux H$^+$ to the intermembrane space from the mitochondrial matrix (Grover et al 2004; Murphy & Steenbergen 2008b). If ischemia persists first, level of ATP further drops and Na$^+/K^+$ ATPase is inhibited. Second, activation of lactate production and ATP hydrolysis cause intracellular acidification (low pH) that triggers Na$^+/H^+$ exchanger (NHE). This results in increased intracellular concentration of Na$^+$ and strives to raise intracellular pH and this would lead to the activation of Na$^+/Ca^{2+}$ exchanger (NCE) and eventual Ca$^{2+}$ overload occurs. Ca$^{2+}$ overload triggers degrading enzymes such as phospholipases, nucleases and proteases which results in damage of membrane integrity (Baines 2009; Halestrap & Pasdois 2009). During reperfusion many factors contribute to the disruption of membrane phospholipids that leads to reperfusion injury, such as inadequate synthesis of ATP, Ca$^{2+}$ overload, production of nitric oxide (NO$^*$), excessive ROS (Crompton 1999; Kutala et al 2007; lady-love et al 2003). Excessive concentration of ATP during reperfusion causes cardiomyocytes hyper-contraction, membrane damage and necrosis (Piper et al 2004; Piper et al 2006). The following reasons lead to the rapid opening of the mitochondrial permeability transition pores (MPTP): Ca$^{2+}$ overload, oxidative stress due to excessive ROS generation, sudden recovery of intracellular pH. MPTP in turn causes the hyper-contraction of cardiomyocytes which results in apoptotic and necrotic death (DI Lisa & Bernardi 2009; Piper et al 2004; Piper et al 2006; Ruiz-Meana et al 2007).

1.4.3 Factors involved in Reperfusion Injury

Impairment to the cell membrane may in turn induce the discharge of more free bases. ROS and calcium (Ca$^{2+}$) level increases, which lead to
mitochondrial Ca\textsuperscript{2+} accumulation, particularly when oxygen is reintroduced (reperfusion) (Murphy & Steenbergen 2008b). Subsequently, ATP generated during reperfusion causes partial reduction of oxygen to water producing ROS, which causes damage to the electron transport chain (Giorgio et al 2005; Zweier & Talukder 2006). This increase in mitochondrial ROS and Ca\textsuperscript{2+} overload opens the mitochondrial permeability transition pore (MPTP), additionally MPTP opening increases with a decrease in mitochondrial membrane potential (\(\Delta \psi \text{m} \)) which further compromises cellular energetic; rupture of the plasma membrane and cell death occurs (Murphy & Steenbergen 2008b). This phenomenon of increase in cardiomyocytes death and infarct size during reperfusion after ischemia is called as myocardial Ischemia/Reperfusion (I/R) injury. Moreover oxidative stress and inflammation are the potential mediators of I/R injury.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{Factors_affecting_Ischemia_Reperfusion_injury.png}
\caption{Factors affecting the Ischemia/Reperfusion injury}
\end{figure}
1.4.4 Mitochondrial Permeability Transition Pore

MPTP is a high conductance mega channel. MPTP links the outer and inner mitochondrial membranes. When it is opened, it allows the contact between the inner mitochondrial matrix and the cytoplasm (Hunter et al 1976). The molecular characteristics of the protein(s) forming this MPTP are yet to be found. Studies have been reported that the MPTP is formed by the voltage-dependent anion channel (outer mitochondrial membrane), adenine nucleotide transporter (inner mitochondrial membrane), and cyclophilin D (mitochondrial matrix). Ca\(^{2+}\) and inorganic phosphate (Pi) mediates the binding of the cyclophilin D to the adenine nucleotide transporter and facilitates the opening of MPTP (Di Lisa & Bernardi 2009).

1.4.5 Mitochondrial Permeability Transition Pore Opening

MPTP opening is the major factor during reperfusion injury. MPTP opening has been contributed to the release of cytochrome c, Bax translocation that raise ROS generation, which results in cell death (Di Lisa & Bernardi 2006; Di Lisa et al 2011). During severe ischemia the mitochondria gets de-energized due to the obvious decrease in intracellular pH (low pH) and the possibility of MPTP opening is very much reduced (Nicollì et al 1993). Also mitochondrial Ca\(^{2+}\) uptake is simultaneously reduced due to the low pH, which further triggers calcium efflux through the activation of mitochondrial NHE and subsequent NCE from the mitochondrial matrix (Duchen 2000). Glycolysis inhibition and pyruvate production also result due to low pH resulting in a decreased supply of H\(^+\) to respiratory complex chain. Hence, during ischemia, low pH is the key to prevent MPTP opening.

Extremely different conditions are created at the start of reperfusion, it may or may not recover mitochondrial membrane potential. If depolarization of mitochondrial membrane occurs, then permanent opening of
MPTP and sudden recovery of intracellular pH, increased ROS generation, Pi Ca\(^{2+}\) overload and decrease in the levels of NO\(^{-}\) generation (Di Lisa et al. 1995; Piper et al. 1998). The possibility of MPTP opening is facilitated by multiple factors such as bursts of ROS, Ca\(^{2+}\) overload and high pH at the start of reperfusion is the direct effect of reperfusion. Reperfusion also mediates the indirect effect in the opening of MPTP through activation of calpain and phospholipase (Inserte et al. 2011; Shulga & Pastorino 2006).

### 1.4.6 Consequences of Mitochondrial Permeability Transition Pore Opening

MPTP opening is of two types: one is transient or intermediate, and another is long-lasting opening, this variation depends on the balance between the cellular inducers and inhibitors (Petronilli et al. 2001). Transient MPTP opening is reversible and it has been suggested to be necessary for the cardioprotection. It is also involved in some physiological functions such as the transient intracellular generation of ROS and NAD\(^{+}\) traffic (Bernardi & Petronilli 1996; Di Lisa & Ziegler 2001; Wang et al. 2008). In fact, during preconditioning for cardioprotection transient ROS is produced which increases the possibility of transient opening of MPTP (Hausenloy et al. 2009; Lim et al. 2007). It is not necessary that transient/intermediate pore opening causes apoptosis. Long lasting pore formation (opening) is irreversible and it involves numerous alterations in cellular bioenergetics. So it increases the mitochondrial permeability and allows the solutes or ions with less than or equal to 1.5 kDa molecular weight. This causes the swelling of mitochondrial matrix and loss of membrane potential. This alteration causes F\(_{0}\)F\(_{1}\)ATPase to trigger hydrolysis instead of its usual ATP synthesis and resulted in cell death (Di Lisa et al. 2007; Griffiths & Halestrap, 1993, 1995; Halestrap et al. 2004). The major consequence of long-lasting MPTP opening is destabilization of mitochondrial membrane potential, depletion of ATP and NAD\(^{+}\), swelling of
mitochondrial matrix, release of accumulated Ca\textsuperscript{2+}, outer mitochondrial membrane rupture and cytochrome c release, which further activates caspase cascade and blocks the electron flow via electron transport chain (Bernardi et al 2006; Di Lisa et al 2001; Heusch et al 2010) (Di Lisa & Bernardi 2009). Many reports have been suggested that long lasting MPTP opening is irreversible and it leads to cell death (Batandier et al 2004; Crompton 1999; Griffiths & Halestrap 1995).

1.4.7 Metabolic Derangement in Cardiomyocytes During Ischemia/Reperfusion

During ischemia, the intracellular concentration of H\textsuperscript{+} ions, Ca\textsuperscript{2+} and Na\textsuperscript{+} increases and ATP level decrease which causes increase in osmotic loading and mitochondrial injury. In fact oxygen deficiency occurs after ischemia and this causes damage to mitochondrial complexes I and III. The accumulated intracellular H\textsuperscript{+} ion causes inactivation of glyceraldehyde dehydrogenase and cytochrome oxidase inhibit oxidative phosphorylation and anaerobic glycolysis is inhibited by NADH (Jennings & Reimer 1991; Lesnefsky et al 2001). For transient maintenance of mitochondrial membrane potential during ischemia mitochondrial ATPase uses ATP and this cause the fall in the level of ATP during myocardial ischemia. The intracellular concentration of Na\textsuperscript{+} is increased during myocardial ischemia via three mechanisms: first, through stimulating Na\textsuperscript{+}– H\textsuperscript{+} exchanger. Second, through un-inactivated Na\textsuperscript{+} channels and third, through inhibition of Na\textsuperscript{+} efflux via the Na\textsuperscript{+}– K\textsuperscript{+} pump (Inserte et al 2006; Inserte et al 2002; Murphy & Steenbergen 2008a). This intracellular increase in Na\textsuperscript{+} triggers influx of Ca\textsuperscript{2+} through Na\textsuperscript{+}– Ca\textsuperscript{2+} exchanger in ischemic myocytes. Along with this sarcoplasmic reticulum Ca\textsuperscript{2+} uptake and Ca\textsuperscript{2+} efflux through the sarcolemmal Ca\textsubscript{2+} pump is decreased. Thus, during ischemia increase in intracellular Ca\textsuperscript{2+} concentration is kept moderate. Moreover acidosis occurs due to increase in
intracellular H\textsuperscript{+} ion concentration and it inhibits Na\textsuperscript{+}–Ca\textsuperscript{2+} exchanger. Along with this influx of cytosolic Ca\textsuperscript{2+} into mitochondrial membrane occurs during ischemia to maintain mitochondrial membrane potential by the utilization of ATP (Inserte et al. 2002; Murphy & Steenbergen 2008a). Severe ischemia stimulates sarcolemmal injuries as a result of an increase in long-chain Acyl-CoA and Ca\textsuperscript{2+} activated proteases (Jennings & Reimer 1991). This sarcolemmal damage stimulates the necrotic cell death. Necrotic cell death can be salvaged by reperfusion but it produces irreversible cell death in case of severe ischemia. In contrast to ischemia, reperfusion inhibits acidosis-mediated inhibition of the Na\textsuperscript{+}–Ca\textsuperscript{2+} exchanger, resulting in a huge influx of Ca\textsuperscript{2+} into cardiomyocyte (Inserte et al 2002; Murphy & Steenbergen 2008a). The sudden influx of huge amounts of Ca\textsuperscript{2+} into the mitochondria at the start of reperfusion triggers calpain that causes dysfunction of Na\textsuperscript{+}–K\textsuperscript{+} pump. These results in delay of the rescue of mitochondria from the Ca\textsuperscript{2+} overload and destabilized ionic homeostasis (Inserte et al 2002; Murphy & Steenbergen 2008a). In addition, swelling of cardiomyocytes occurs due to influx of water due to increase in cytosolic osmolarity by glycogen and high-energy phosphate catabolism during ischemia (Jennings & Reimer 1991). Re-introduction of oxygen to the mitochondria restores ATP generation and also increases the production of free radicals such as ROS form the complexes I and III (Turrens 2003; Zweier & Talukder 2006). Hence the increase in ROS generation and Ca\textsuperscript{2+} overload are considered to be a major source for the MPTP opening (Zorov et al 2009). By the inhibition of MPTP using pharmacological inhibitors during reperfusion the size of the infarct can be decreased.

1.4.8 MPTP opening and necrosis of cardiomyocytes

MPTP is the mega channel in the inner mitochondrial membrane which allows molecules of < 1.5 kDa when it opens. I/R and anti-cancer
agents causes irreversible opening of MPTP that lead to necrotic cell death (Zorov et al 2009). The extent of the MPTP opening was studied using radiolabelled 2-deoxyglucose showed that during ischemia MPTP remain closed and opens only after 5–10 min of reperfusion in an isolated rat heart (Griffiths & Halestrap 1995). The time taken for the opening of MPTP is consistent with the observations of Ca\(^{2+}\) overload, ATP depletion, and increase in ROS production. The sudden influx of Ca\(^{2+}\) and uncontrolled ROS production occurs only during reperfusion (Griffiths & Halestrap 1995; Murphy & Steenbergen 2008a; Zweier & Talukder 2006) (Turrens 2003). Some of the pharmacological inhibitors and inhibition of Cyp D decrease the infarct size in \textit{in-vivo} after I/R injury. Thus, it is clear that MPTP plays a key role in I/R induced cardiomyocyte necrosis (Argaud et al 2005; Baines et al 2005; Gomez et al 2008; Nakagawa et al 2005).

1.5 SARCOPLASMIC RETICULUM

In myocytes instead of endoplasmic reticulum (ER), sarcoplasmic reticulum (SR) is present. The difference between SR and ER is the structure of proteins. This structural difference made their functions different. The function of ER is to synthesize molecules, whereas the function of SR is to store and pump calcium ions. SR has a major role in excitation-contraction coupling (Ernster & Schatz 1981; Henze & Martin 2003).

1.5.1 Mitochondria-Associated ER membrane Role in Calcium Homeostasis

The ER is the most significant storage site of calcium, and there is a significant interplay between the mitochondrion and ER with regard to calcium. Mitochondria-associated ER membrane (MAM), comprises about 20% of the mitochondrial outer membrane, the ER and mitochondria are separated by a distance of about 10–25 nm and held together by protein
tethering complexes (Rizzuto et al 2009). The enzymes present in MAM have shown to be involved in phospholipid exchange, and Ca$^{2+}$ signaling (Rizzuto et al 2009). Mitochondria transiently stores calcium and maintain cell calcium homeostasis (Brady et al 2005). Mitochondria act as cytosolic buffers by its ability to rapidly uptake calcium and release it later for maintaining calcium homeostasis and to inhibit the oxidative stress (Brighton & Hunt 1974). The calcium is taken up into the matrix by a calcium uniporter on the inner mitochondrial membrane and is primarily driven by the mitochondrial membrane potential (Brady et al 2005). The release of this calcium back into the cell's interior can occur via a sodium-calcium exchange protein or via "calcium-induced-calcium-release" pathways. This can initiate calcium spikes or calcium waves with large changes in the membrane potential. Ca$^{2+}$ influx to the mitochondrial matrix is associated with the regulation of respiratory bioenergetics. It is by allowing the electrochemical potential across the membrane to rapidly "pulse" from membrane potential-dominated to pH-dominated, eventually it leads to reduction of oxidative stress (Schwarzländer et al 2012).

### 1.6 CASPASE CASCADE

Alterations in mitochondrial membrane potential are intimately linked to the modulation of caspases (cysteine protease) activity. Caspases belongs to the cysteine protease family that cleaves their substrates after aspartate residues. Moreover caspases are always synthesized as zymogens which are inactive. During the initiation of apoptosis only these inactive zymogens are cleaved into subunits. Then these cleaved caspases are activated by forming heteromeric complexes with death factor receptor. Caspases can be functionally classified into three types. Caspase-1, -4, -5, -11, -12, and -13 are type-I caspases. The main functions of these caspases are processing of cytokines. Caspase-3, -6, and -7 are type-II caspases and they are called as
executioner or effector caspases. The main function of these effector caspases is to cleave the crucial cellular protein substrates that lead to cell death. Caspase-2, -8, -9, and -10 are type III caspases and they are termed as initiator caspases. The main role of these initiator caspases is to activate downstream executioner caspases and results in the amplification of caspase activity (Wolf & Green 1999).

There are two different pathways which are involved in caspase activation that lead(s) to apoptosis (programmed cell death) /cellular membrane PS (Phosphatidyl serine) exposure and genomic DNA cleavage (Lin & Maiese 2001). First, extrinsic pathway occurs before modifications of mitochondrial membrane potential. This pathway is initiated by the activation of death receptors at the cell surface. The death receptor activation further activates caspase-8 and -10. Activated caspase-8 further cleaves Bid and translocate to mitochondria. It consequentially releases cytochrome c via the Bax subfamily of Bcl-2 proteins (Tang et al 2000). Consequently, degradation of genomic DNA and exposure of membrane PS can proceed through the activation of executer caspase 3 and caspase-1 following caspase-8 activation (Takahashi et al 1999). Second, intrinsic pathway is mediated by caspase-9 (initiator caspases) after the release of mitochondrial cytochrome c. The released mitochondrial cytochrome c binds to Apaf-1 and then activates executioner caspases-3 and-7 (Cain et al 2000).

### 1.7 PROTEIN KINASES INVOLVED IN MITOCHONDRIAL SIGNALING FOR CARDIOMYOCYTE PROTECTION

#### 1.7.1 Akt

Protein Kinase B/Akt has three isoforms such as Akt1, Akt2, and Akt3. All three isoforms have approximately 80% homology; however, each isoform is encoded by a different gene locus. From the total Akt, Akt1 and
Akt2 were the major Akt’s in cardiomyocytes (Matsui & Rosenzweig 2005). Activation of cytokine receptors, tyrosine kinases receptor leads to the stimulation and production of phosphatidylinositol 3, 4, 5-triphosphate (PIP3) in the cell membrane through activation of phosphatidylinositol 3'-kinase (PI3K). The accumulation of PIP3 leads to swift Akt to the plasma membrane and then Akt is partially activated by phosphoinositide-dependent protein kinase-1 (PDK1) -mediated phosphorylation at Thr308. For the full activation of Akt it requires phosphorylation of Ser473 by PDK2. Then the phosphorylated Akt is supposed to release from the membrane and binds with the substrates in the cytoplasm, mitochondria and nucleus (Bijur & Jope 2003; Kobayashi et al 2008; Matsui & Rosenzweig 2005; Miyamoto et al 2007). In response to the receptor activation Akt has been translocated to the mitochondria in endothelial cells/fibroblast cells and cardiac cells. Insulin or Insulin-like growth factor-1 activates Akt signaling pathways in a minute, which have been demonstrated in mitochondrial fractions. Studies have shown increased levels of total and phospho-Ser473-Akt in the mitochondrial fraction when neonatal rat ventricular myocytes induced with leukemia inhibitory factor (LIF) for 10 min (Matsui & Rosenzweig 2005). But the mitochondrial translocation of Akt in in-vivo rat heart models has not been established. A numerous receptor-mediated protective interventions are responsible for tolerance of necrosis and these receptors utilizes Akt signaling pathways for cardioprotection against necrosis. Ischemic preconditioning (IPC) and cytokine receptor activation causes reduction of infarct size through phosphorylation of Akt and this protective effect is abolished by deletion of either PI3K inhibitors or PDK1 gene deletion (Downey et al 2007; Miki et al 2007). Prolonged activation of Akt also limited the infarct size in ischemia/reperfusion injured heart (Budas et al 2006). The role of each isoforms of Akt remains unknown. However Akt isoforms showed sex differences in their functions. Knockdown of Akt1/2 in male and Akt3 in
female cardiomyocytes showed abolition of protection against I/R injured necrosis (Cao Z et al 2008).

Akt contributes its protective effect during I/R by IPC by two ways. First, protection in triggering phase during which the receptors get activated and offers resistance to cardiomyocytes for the successive insults. Second, protection in mediator phase in this Akt mediates the suppression of concomitant I/R injury (Cao et al 2008; Downey et al 2007). The protective effect of IPC was eliminated by blocking of PI3K either before ischemia or at the time of reperfusion. In triggering phase Akt contributes its protective effect in cytosol. Several studies reported that the PI3K activates Akt and the activated Akt further activates ERK, eNOS, protein kinase G and these activated proteins eventually activates mitochondrial ATP-sensitive K\(^+\) channel (mK\(_{\text{ATP}}\)) (Cohen et al 2007; Downey et al 2007; Philipp et al 2006). Of all the receptors involved in IPC protective function d-opioid receptor and bradykinin B2 receptor are involved in ROS production via mK\(_{\text{ATP}}\), whereas the adenosine receptor is independent of mK\(_{\text{ATP}}\) channel activated by PI3K/Akt signaling (Griffiths & Halestrap 1995). In contrast to triggering phase of IPC protection, mediator phase of IPC supposed to be showing its protective role in mitochondrial compartment. During reperfusion Akt gets phosphorylated after IPC and activates translocation of HK (hexokinases), an Akt substrate to mitochondria and blocks the opening of MPTP at the start of reperfusion (Hausenloy et al 2003; Moon et al 2006; Zuurbier et al 2005). Apart from the above receptor and the mentioned mechanisms several targets of Akt involved inside and outside for the mitochondrial protection are yet to be elucidated.
1.8 ERYTHROPOIETIN

1.8.1 The Cell Biology of Erythropoietin

Erythropoietin (EPO) is a glycoprotein hormone/cytokine that is produced primarily in the peritubular interstitial cells of the kidney (Hanlon et al 2005) with a molecular weight of 30.4 kDa and its gene is located on chromosome 7q11-22. It comprises of 4 introns and 5 exons and encodes 193 amino acid precursor protein. This consists of glycosylation of 3 N-linked (at Asn-24, Asn-38 and Asn-83) and one O-linked (Sr-126) amino acids, cleavage of 27 amino acid sequence, Arginine residue (Arg-166) removal of the C-terminal end yields 165 amino acid circulating EPO. Four anti-parallel $\alpha$ – helices formed the tertiary structure. In 1997, EPO was purified from aplastic anemic patients (Miyake et al 1977). In 1985, DNA probes were synthesized from tryptic fragments of urinary EPO for cloning and isolation of the human EPO gene. In 1987, recombinant human EPO was manufactured and used to treat anemia of chronic renal failure (Eschbach et al 1987) (Winearls et al 1986). In response to hypoxia, EPO is secreted from the kidney to maintain normal erythropoiesis and oxygen levels in healthy adults. EPO concentration in plasma ranges from 15 - 25 mU/L to maintain the optimal picomolar range of base level of EPO secretion. Hypoxic stimulation increases plasma levels by 50 - 100 folds (Al-Huniti et al 2004). Initially, it was reported that EPO is produced only in the interstitium of the cortex and outer medulla of kidney. Studies have been reported that EPO is also produced in fibroblast-like type I interstitial cells in transgenic mice (Maxwell et al 1993a). In addition, EPO is also produced in other sites such as brain, endometrium, epididymis (Masuda et al 1994), (Yokomizo et al 2002), (Kobayashi et al 2002). The function of EPO at these sites are yet to elucidated, though fetal EPO production is necessary for the physiological role, EPO produced in these locations remains uncertain, but fetal production
at multiple sites may be essential for normal growth and development (Juul et al 1998).

**Figure 1.7 Primary structure of Erythropoietin**

**1.8.2 Regulation of EPO production**

The expression of EPO gene is regulated by the HIF. HIF is oxygen-sensitive transcription factor consists of 3 regulatory sub-units (HIF-1α, HIF-2α, and HIF-3α) and HIF-1ß sub-unit (Wang & Semenza 1993). The role of these HIF isoforms remains unclear. Initially HIF was purified from human hepatoma (HepB3) cell line, purified HIF was found to be linked to HRE, which is 3’ regulatory element of 18-nucleotide fragment. It was found that the regulation of HRE is mostly depending on the HIF-2 and not HIF1 in 51 siRNA knockdown studies (Warnecke et al 2004). Inhibition of HIF-2 by with Cre-LoxP results in the decreased production of EPO in liver and
deletion of HIF-2 results in further decrease in production of physiological EPO (Rankin et al 2007). Studies suggested that this decrease in EPO concentration is mainly because of binding of HIF-2 with HRE (Ratcliffe, 2007). During cobalt administration iron act as a cofactor for prolyl-4-hydroxylase and it mimics the hypoxia effect on HIF-1 activation. IPC using Cobalt in rat models showed increased activation of HIF-dependent proteins such as VEGF, heme-oxygenase-1 (HO-1) and EPO and thus results in a decrease in injury caused by I/R in renal cells, thus HIF dependent EPO production plays a significant role in renal protection. In addition, transgenic heterozygotic HIF -/+ mice, which express HIF-1 only in small amounts, but continuously expressing the same showed increased resistance to IPC. This may result in significantly increased production of EPO compared to HO-1 and VEGF in the kidney. This evidence supports the above studies and showed the importance of EPO in IPC effect. Apart from this activation of HIF leads to modifying the expression of numerous cytokines and mediates the increase in resistance (adaptive response) to ischemia and stress. This adaptive response is mediated by some of the mediators such as glucose transporters (GLUT1), glycolytic enzymes, vascular endothelial growth factor (VEGF), transferrin and numerous genes involved in cell viability, proliferation and differentiation (Iyer et al 1998).

1.8.3  EPO role in Hematopoietic Tissues

In the reticuloendothelial system new RBC’s are endlessly produced during the lifetime because of aging and erythrocyte death. Erythroid cell maturation comprises of different stages, each stage requires activation of several growth factors for cell viability, proliferation and differentiation (Kaushansky 2006). EPO’s effect on erythroid components is mediated by binding of EPO receptor on erythroid precursors such as erythroid colony-
forming units (CFU-E) and intermediate-stage erythroid burst-forming units (BFU-E). These are differentiated from pluripotent stem cells. Studies have shown that erythropoiesis is abolished; EPO-R knock-out mice moreover showed the presence of BFU-E and CFU-E in fetal liver tissue, hence the EPO is not necessary for the differentiation (Wu et al 1995). The regulation of erythropoiesis is mainly because of EPO receptor expression. The EPO receptor expression is depending on particular phases of differentiation, the erythroid progenitor cells may die in the absence of EPO (Boyer et al 1992).

In murine erythroid progenitor cells, EPO effectively increases the cell proliferation and induce cell cycle entry of dormant cells (Miller et al 1999). In human erythroid progenitor cell line (HCD-57), EPO efficiently maintained the cell viability by increasing the expression of antiapoptotic proteins such as Bcl-XL (Silva et al 1996).

1.8.4 EPO Role in Non-Hematopoietic Cells

Erythropoietin (EPO) is a 30 kDa protein. Though EPO is hypoxia-induced cytokine which is a hematopoietic hormone, an erythroid precursor causes cell proliferation, differentiation and stimulates erythropoiesis. Apart from erythropoiesis, EPO has a cardio protective effect which increases the number of capillaries and mature vessels in infarcted hearts (Boucher et al 2008; van der Meer et al 2005) and up regulates the expression of angiogenic cytokines such as VEGF and angiopoietin-1. The EPO gene expression is regulated mainly by hypoxia (Maxwell et al 1993b; Wenger 2000). Apart from playing a role in hematopoiesis, the EPO also has a protective role in other systems (Henry & Spivak 1995). Several reports have shown their efficacy in heart myocytes, ovarian, glial cells, brain and retinal diseases (Grimm et al 2002; Schöffel et al 2008a; Siren et al 2001). Indeed, protective
effects of EPO have been described in models of cerebral ischemia (Bernaudin et al 1999; Sirén et al 2001), retinal degeneration (Junk et al 2002) Alzheimer's disease (Chong et al 2005; Vesey et al 2004a), renal injury 40, Parkinson’s disease (Xue et al 2007), hepatic ischemia (Sepodes et al 2006) and myocardial ischemia. On these tissues EPO is linked to promoting cell proliferation and differentiation, angiogenesis or inhibition of apoptosis (Schöffel et al 2008b). A study in rats subjected to cerebral ischemia showed a significant reduction in brain infarct size (Brines et al 2000; Siren et al 2001). The specificity and biological relevance of these changes were demonstrated by the observation that the neutralization of endogenous EPO with soluble EPO-receptor (EPO-R) augments ischemic brain damage (Sakanaka et al 1998b). It is now well known that EPO increases red blood cell mass mainly by altering the balance between erythropoiesis and apoptosis.

1.8.5 Erythropoietin Receptor and Signaling Pathways

To demonstrate the specific binding of EPO to erythroid lineage cells, previously cDNA investigators used radiolabelled EPO before cloning of the EPO receptor (D'Andrea et al 1990). They found out 200 EPO receptors on the purified erythroid progenitor cell surface. This EPO receptor resembles the characteristic of GM-CSF, G-CSF and interleukin-3 (Groopman et al 1989). By using radiolabelled EPO affinity cross-linking experiments they found out two cross-linked EPO receptor complexes, following crystallization studies EPO receptor dimerisation has been confirmed (Matthews et al 1996). Recombinant plasmids from the MEL cDNA library were used for cloning of the murine EPO receptor into the COS cells and radiolabelled EPO were used to screen the transfected cells. The cloned EPO receptor consists of 507-amino acid polypeptide, single membrane spanning domain and resembles the
homology of interleukin 2 (D'Andrea et al 1990). In addition the extracellular
domain consists of ~200 amino acids, in which 100 amino acid sequence is
believed to be derived from a type III fibronectin subdomain (Bazan 1990).
Moreover EPO receptor possesses a WSXWS sequential in subdomain on the
proximity of the membrane which is essential for the maintenance of
membrane stability and folding. In the first domain it consists of cysteine,
which is held together by disulphide bonds (Yoshimura et al 1992).

The EPO’s erythropoietic effects are achieved through a 66-78 kDa
membrane-spanning protein that is a member of the cytokine superfamily of
receptors (Fisher 2003). Upon binding to its receptor, EPO exerts its
intracellular effects through tyrosine phosphorylation of a number of proteins
(Fisher 2003). However the EPOR does not possess endogenous tyrosine
kinase activity (Miura et al 1994b). Instead, upon EPO binding, the preformed
dimeric receptor undergoes a conformational change which in turn allows the
activation of Janus kinase 2 (JAK2) which is constitutively associated with
the EPOR close to the transmembrane portion of the receptor (Livnah
et al 1999). JAK2 then phosphorylates signal transducer and activator of
transcription factors (STATs), as well as 8 tyrosine residues in the
cytoplasmic domain of the EPOR which act as docking sites for proteins
containing Src homology 2 (SH2) domains (Fisher 2003). After binding,
the SH2-domain containing protein is tyrosine phosphorylated and
subsequently activated. The activation of phosphatidylinositol-3 (PI3) kinase
(He et al 1993), extracellular signal related kinases (ERK1/2) and
phospholipase C (Miura et al 1994c) is commonly seen following EPOR
activated in this manner. A summary of the common signaling pathways
activated by EPOR signaling is shown in Figure 1.8.
After binding with its receptor, EPO signals through multiple kinases pathways, like mitogen-activated protein kinases (MAPK) and Janus kinase/signal transducers and activators of transcription (JAK2-STAT5) tyrosine kinases, phosphatidylinositol 3-kinase[PI(3)K] (van der Meer et al 2004). In these tissues EPO exerts various effects such as apoptosis inhibition, cell proliferation and cell differentiation (Schöffel et al 2008b).

### 1.8.5.1 Akt Signaling pathway

Initially phosphorylation of Akt at Ser-124 and Thr-450 occurs independently of cell stimulation or PI3K activation, and most likely renders Akt competent to undergo activation upon exposure of cells to extracellular stimuli. GF (Growth Factor) stimulation of PI3K leads to increased intracellular levels of PI3, 4P and PI3, 4,5P. Binding of these lipids to the Akt PH (Pleckstrin homology) domain results in Akt translocation from the
cytoplasm to the plasma membrane; in addition, the interaction of the PH domain with the lipid products of PI3K causes a conformational change in Akt that renders Akt accessible to phosphorylation at Thr-308 and Ser-473. Phospholipids also increase the ability of the PDK-1 which may be complexed with PRK-2, a fragment of PRK-2, or other related proteins-to phosphorylate Akt. The final step in Akt activation appears to be the phosphorylation of Thr-308 and Ser-473 by PDK-1 (Datta et al 1999).

1.8.5.2 p38 MAPK signaling pathway

During apoptosis, DNA damage, ischemia, heat shock, UV irradiation and oxidative stress occur and p38 MAPK is activated (Johnson & Lapadat 2002), (Dent et al 2003). In fact, p38 MAPK is activated by phosphorylation at the active site via Thr180/Tyr182 and this phosphorylation is via the upstream of MKK3, MKK4 and MKK6 (Derijard et al 1995), (Zarubin & Jiahuai 2005). Interestingly p38 MAPK produce different substrates depends on the different stimuli, the protein kinases such as MnK, MAPKAPK2; death/survival molecules like Bcl2, caspases; transcription factors such as ATF2, MEF2, and cell cycle control factors like cyclin D1 (Alvarado-Kristensson et al 2004; Wood et al 2009; Zhao et al 1999). Accordingly p38 MAPK signaling promotes cell death and senescence in some cancer cells and at the same time p38 MAPK has been shown to enhance cell survival, cell growth (yeast and mammals cell lines), and cell differentiation (3T3-L1 cells into adipocytes and PC12 cells into neurons) in some cell lines (Zarubin & Jiahuai 2005). EPO stimuli activate p42/p44 MAPK and act as survival signaling pathway during I/R in cardiac cells (Schulman et al 2002; Zu et al 1997).
1.8.6 Cardio Protective Role of EPO

The cardio protective functions of EPO are mediated by EPO-R and its signaling pathways (Ueda et al 2008). In support of this notion that EPO, an essential component required for increasing hematocrit, exerts a protective effect against ischemia in hearts from normoxic rabbits, from birth to 10 days of age (Shi et al 2004). After a century of research and medical use, EPO has more therapeutic approaches than ever in history. After cloning its gene in 1984, EPO obtained FDA license for clinical use in 1989. EPO and its analogues are mainly used for treatment of the anemia associated with chronic renal failure and malignancies. Regarding research undertaken in the past 15 years, tremendous efforts were made for improvement of bioactivity, half-life and alternative application. Today, there are human cell-lined derived EPO, SEP, CEPO, CERA and drugs which are linked to different pathways of signaling. This detection offered approaches in the treatment for apoplexia and cardiac infarction and even in preventive treatment of cardiovascular diseases which led to an interest of manifold subject categories (Schöffel et al 2008b).

1.8.7 Role of EPO in Angiogenesis

Ischemic heart disease is characterized by a reduction in blood supply to the myocardium. It has been suggested that angiogenesis, the formation of new capillaries from pre-existing vessels may be beneficial in heart disease through the restoration of perfusion of heart (Lekas et al 2004). Angiogenesis have been shown to improve heart function in dilated cardiomyopathy, pressure-overload induced hypertrophy (Friehs et al 2006), and following MI (Fukuda et al 2006). Because of this, a number of clinical studies have investigated the effectiveness of pro-angiogenic factors in ischemic heart disease (Losordo et al 1998). Interestingly, endothelial cells express EPOR and are known to proliferate in response to EPO treatment.
(Anagnostou et al 1994) and deletion of the EPO or EPOR is associated with angiogenic defects during development (Kertesz et al 2004). EPO increased capillary growth, in cultured human myocardial tissue, to a level similar to that of VEGF. Interestingly, one study in mice has provided evidence that EPOR activation may regulate VEGF expression during peripheral ischemia. EPO significantly improved ventricular function while increasing angiogenesis in the peri-infarct myocardium. EPO analogue darbepoietin alfa increased capillary density and improved heart function (Nakano et al 2007).

1.8.8 The Novel Role of Erythropoietin in the Nervous System

In humans, rodents, and primates EPO and EPOR are expressed in the nervous system. EPO is expressed in the cortex, medulla, capsula interna and hippocampus in the mouse model (Digicaylioglu et al 1995). EPOR has been demonstrated in cultured rat cortical neurons by PCR and immunofluorescence studies (Morishita et al 1997). Both EPO and the EPO receptor presence were demonstrated in astrocytes and neurons in humans. However the expression level varies depends on the gestational age (Juul 2000).

Previous studies have shown the EPO protection in neural cells during cerebral ischemia. EPO has shown protection against ischemia-induced learning disability and prevent hippocampal neuron degeneration in gerbils, which was subjected to occlusion of common carotid arteries (Sakanaka et al 1998a; Sakanaka 1998). In-vivo beneficial effects of EPO were shown in protection of ischemic brain injury (Grasso et al 2005; Matsushita et al 2003; Sadamoto et al 1998).
1.9 AIMS

Despite the above mentioned studies, there is no clear understanding of EPO’s cardio-protective mechanism, i.e. the signaling pathways by which it protects the cardiomyocytes from Hypoxia/Reperfusion (H/R) injury. This study serves to elucidate this mechanism by investigating the role of EPO in preventing H/R injured cell death in H9C2 cells and neonatal cardiomyocytes.

The main objectives of our present study are

1) To evaluate the protective role of EPO against apoptosis and necrosis in H/R injured myocytes.

2) To evaluate the mechanistic pathway of EPO in H/R injured myocytes.

3) To evaluate the downstream effectors of EPO in H/R injured myocytes.