CHAPTER 3

Review of Literature
3. REVIEW OF LITERATURE

Acute coronary occlusion will be the leading cause of mortality by 2020, according to World Health Organization report (Murray and Lopez, 1997). Despite recent advances in treatment of coronary artery diseases, cardiovascular disorders continue to rank as the most frequent cause of death worldwide (Cohen et al., 2000). Prompt reperfusion of ischaemic myocardium is required to salvage it (Topol et al., 1992). Reperfusion of ischaemic myocardium is not without risk (Piper et al., 2004) as it is associated with further increase the extent of myocardial injury and known as reperfusion injury (Baxter and Ebrahim, 2002). Moreover, a delay to institute reperfusion deprives the myocardium from its beneficial effects. Thus a lot of attention has been focused on to understanding the adaptive mechanism(s) that make the myocardium (a) more resistant to sustained ischaemia of longer duration and (b) to restore its viability on reperfusion (Yellon and Baxter, 2000; Bolli et al., 2011).

3.1 Concept of Preconditioning

Repeated short episodes of ischaemia and reperfusion have been demonstrated to make myocardium transiently more resistant to the deleterious effects of prolonged ischaemia (Moroko et al., 1971). This paradoxical form of myocardial adaptation has been termed as ischaemic preconditioning (Murry et al., 1986). Ischaemic preconditioning is not a unique phenomenon of cardiac myocytes. It is reported to occur in liver (Nilsson et al., 2000), brain (Sharp et al., 2004), lung ( Featherston et al., 2000) and skeletal muscles, (Cheng et al., 2000). The protective effect of ischaemic preconditioning is transient and wanes off gradually within 1 to 3 hours in different species (Baxter and Ebrahim, 2002). Moreover, cardioprotective effect of ischaemic preconditioning re-appears, of its own, after 24 h (Bolli, 2000; Ebrahim et al., 2011).
The early phase of protection has rapid onset and short duration (Sack et al., 1993). It is known as classic preconditioning or first window of protection (FWOP) (Murry et al., 1986, Baxter & Ebrahim 2002). The delayed phase of protection occurring many hours later and lasting much longer has been termed as second window of protection (SWOP) (Bolli et al., 1998, 2011; Bolli, 2000).

Many pharmacological interventions such as endotoxin (Thornton et al., 1993) monophosphoryl lipid A (Yao and Gross, 1993) interleukin –1 (Brown et al., 1990) α1 adrenoceptor agonist (Tsuchida et al., 1994) adenosine A1 receptor agonist (Thornton et al., 1992) bradykinin (Vegh et al., 1991) morphine (Liang and Gross, 1999), endothelins-1 (Hide et al., 1995) and angiotensin (Sharma and Singh, 1999) produce ischaemic preconditioning-like cardioprotective effect and it has been termed as ‘pharmacological preconditioning’ (Klein et al., 2000; Koji et al., 2003 Yellon and Downey, 2003; Peng et al., 2011).

The occlusion of circumflex coronary artery produces protection of myocardium supplied by left anterior descending coronary artery and this phenomenon is termed as intracardiac preconditioning (Wang et al., 2002). Short occlusion of renal (McClahan et al, 1993; Pell et al., 1998) abdominal aorta (Sharma and Singh; 1999, Weinbrenner et al., 2002, Singh and Sharma, 2004) or mesenteric artery (Gho et al., 1996; Singh and Chopra, 2004) has been documented to protect the myocardium, against ischaemia and reperfusion induced injury. This phenomenon has been termed as ‘remote preconditioning’ (Heusch and Schulz, 2002, Singh and Sharma, 2004) or preconditioning at distant site (Schoemaker and Heijningen, 2000, Weinbrenner et al., 2002).
3.2 Molecular Mechanism of Ischemic Preconditioning (IPC)

The mechanisms underlying the early and late ischemic preconditioning have not been completely understood. The signal transduction mechanism involved in the early phase of IPC, triggers the activation of various endogenous ligands (Baines et al., 1999; Murphy, 2004) such as acetylcholine (Yao and Gross, 1993), nor-epinephrine (Banerjee et al., 1993; Seyfarth et al., 1996), adenosine (Liu et al., 1994; Grube et al., 2011), bradykinin (Goto et al., 1995), angiotensin (Liu et al., 1995; Sharma and Singh, 1999; Joseph, 2010), opioids (Schultz et al., 1995; Peart et al., 2008), endothelin (Wang et al., 1996; Duda et al., 2007) which interact with their respective G-protein coupled receptors and initiate a signaling cascade which involves the activation of Phosphoinositide-3-kinase (PI-3K) (Mocanu et al., 2002) and phospholipase C (Tyagi and Tayal, 2002). Administration of pharmacological agents such as insulin, erythropoeitin have also been shown to activate PI-3K (Lawlor and Alessi, 2001; Nishimoto et al., 2010) which generate phosphatidylinositol 3, 4, 5 triphosphate (PIP₃) from cell membrane lipid, phosphatidylinositol 3,4-biphosphate (PIP₂), leading to activation of phosphoinositide-dependent kinase (PDK1) and subsequent activation of protein kinase B (PKB/Akt) (Stokoe et al., 1997; Hausenloy et al., 2004a). The activated Akt phosphorylate substrates include proapoptotic members of the Bcl-2 family, caspases-9, glycogen synthase kinase -3 beta (GSK-3β) and endothelial nitric oxide synthase (eNOS) (Ferdinandy et al., 2007; Murphy and Steenbergen, 2008). The nitric oxide (NO) generated from eNOS (Muscarì et al., 2004; Prendes et al., 2007) leads to activation of protein kinase G (PKG) via elevation of intracellular cGMP level (Gross et al., 2004; Costa et al., 2005; Cohen et al., 2010). This PKG phosphorylates and inactivates GSK-3β which increase the threshold of
MPTP opening in cardiomyocytes, thereby producing the cardioprotective effect of ischemic preconditioning (Tong et al., 2004; Gross et al., 2004; Hausenloy et al., 2004b; Davidson et al., 2006; Smith et al., 2010). Akt is also known to activate p70S6K (Jonassen et al., 2004). In addition, PI-3K stimulate the activation of mTOR which further activates p70S6K and provide cardioprotection through opening of mitochondrial potassium ATP channel (Murphy and Steenbergen, 2008). Moreover, PI-3K also activates PKC and produces cardioprotection (Tong et al., 2000; Berger et al., 2010).

The activated phospholipase C, through receptor dependent mechanism, catalyze the hydrolysis of PIP\(_2\) into inositol triphosphate (IP\(_3\)) and diacylglycerol (DAG) (Tyagi and Tayal, 2002). The DAG translocates PKC from cytosol to perinuclear membrane leading to its activation (Mitchell et al., 1995; Tong et al., 2004). PKC also appears to be activated by ROS generated by the stimuli of preconditioning (Baines, 1997). It has been reported that PKC provides cardioprotection by opening of mitochondrial ATP-sensitive K\(^+\) channel (mito KATP) (Baines, 1997; Murphy, 2004; Law et al., 2010). Recently, PKC-\(\varepsilon\), as well as PKC-\(\delta\), have been demonstrated to mimic preconditioning due to opening of mito KATP channel (Dreixler et al., 2008; Han et al., 2010). Further, opening of mito KATP channel depolarises inner mitochondrial membrane and reduces mitochondrial calcium entry into the mitochondria, resulting in inhibition of opening of mitochondrial permeability transition pore (MPTP) (Javadov et al., 2003; Hausenloy et al., 2004b) and the consequent decrease in the release of cytochrome c and reduction of apoptotic cell death (Kroemer et al., 1998; Hausenloy et al., 2004b). Moreover, opening of mito-K\(_{\text{ATP}}\) channel leads to influx of K\(^+\) in the mitochondrial matrix (da Silva et al., 2003). The influx of K\(^+\) is associated
with entry of weak acids and water leading to transient swelling of mitochondrial matrix which may improve the generation of adenosine triphosphate (ATP) through electron transport chain (Tanonaka et al., 1999; Xu et al., 2001) which also results in inhibition of MPTP opening (Hausenloy et al., 2002; Halestrap et al., 2007). In addition, opening of mitochondrial K$^{+}_{ATP}$ channel has been shown to cause partial alkalization and a small reduction of transmembrane potential leads to production of bursts of subtonic amount of reactive oxygen species (Cohen and Downey, 2007) which has been implicated as a trigger for early cardioprotective effect of ischemic preconditioning through activation of PKC (Baines et al., 1997; Carroll et al., 2001; Andrukhiv et al., 2006). Ischemic preconditioning signals activate the survival kinases such as p38MAPK, ERK1/2, JAK/STAT pathway which may be responsible for inducing cardioprotection (Hausenloy and Yellon, 2006; Ferdinandy et al., 2007). Preconditioning is well capable of protecting ischemic myocardium by limiting infarct size, reducing myocardial stunning, preventing cardiac arrhythmias or accelerating the recovery of myocardial function after the sustained ischemic insults (Tamura et al., 1997; Pomerantz et al., 2000).

The mechanism of late ischemic preconditioning is initiated by the release of endogenous chemical mediators such as adenosine (Baxter and Yellon, 1999; Takano et al., 2001), nitric oxide donors (Bolli et al., 1998, Bolli, 2000), and opioid receptor agonists (Fryer et al., 1999, 2001), bradykinin (Kostitprapa et al., 2001), reactive oxygen species (Otani, 2004), these substances trigger the activation of complex signal transduction that includes PKC-ε and protein tyrosine kinase (Hattori et al., 2001). PKC activates the Src and Lck tyrosine kinases, leading to tyrosine phosphorylation and inactivation of IκBα (the inhibitor of NF-κB) resulting into
activation of nuclear factor-κB (NF-κB) (Ping et al., 2002; Ludwig, 2004). NF-κB translocates to the nucleus, where it binds to its cognate sequence on the promoter region of target genes, including inducible form of nitric oxide (iNOS) (Bolli et al., 1998; Xuan et al., 2000) and COX-2 (Shinmura et al., 2002) and subsequent generation of cardioprotective factors such as NO, PGI2, and PGE2 (Shinmura et al., 2000), which may account for the delayed cardioprotective effect of ischemic preconditioning. Further, PKC-ε also activates mitochondrial K\textsubscript{ATP} channel (Takashi et al., 1999) and inhibit MPTP in the cardiomyocytes to mediate the cardioprotective effect of delayed ischemic preconditioning (Baines et al., 2003). Ischemic Preconditioning activate JAK1-JAK2 and STAT1/STAT3, this heterodimer unit translocates to the nucleus, where it binds to the promoter of target genes (Yamamura et al., 2003). The combinatorial actions of NF-κB and STAT1/STAT3, result in transcriptional activation of inducible form of nitric oxide (iNOS), cyclooxygenase (COX-2), hemoxygenase (HO-1), and superoxide dismutase (SOD) (Hoshida et al., 2002; Peng et al., 2011) and heat shock proteins (HSP) such as HSP90 (Nayeem et al., 1997) and HSP70 (Nayeem et al., 1997; Liu et al., 2004) leading to the synthesis of the respective proteins which mediate the late effect of ischemic preconditioning (Stein et al., 2004). It has been reported that NO can induce HO-1 which catalyzes the heme to biliverdin and carbon monoxide (CO) (Csonka et al., 1999; Clark et al., 2000). CO has been shown to confer delayed cardioprotection by mediating G-protein coupled receptor via its anti-apoptotic actions (Song et al., 1997). Moreover, NO activates COX-2 and induces the production of PGE\textsubscript{2}, PGI\textsubscript{2} which also mediate the late effect of ischemic preconditioning. HSP-72 confers cardioprotection by increasing ecto-5’nucleotidase activity, an enzyme responsible for adenosine formation in rat hearts (Node et al., 1996; Sakaguchi et al., 2000). Moreover HSP-72
enhances SOD activity, resulting in decrease of mitochondrial oxidative stress and the process of apoptosis (Yamashita et al., 1998; Suzuki et al., 2002). Although the role of aldose reductase in late ischemic preconditioning is controversial but may be related to inhibition of lipid peroxidation (Shimura et al., 2002). Apart from this, integral membrane proteins localized in the inner mitochondrial membrane i.e. uncoupling proteins (UCPs) are potentially vital to elicit the cardioprotective effect of delayed ischemic preconditioning (Bienengraeber et al., 2003). Moreover, UCP overexpression confers cardioprotection by reducing oxidative stress via diminished calcium overload and reduced ROS generation at reperfusion (Teshima et al., 2003; Otani et al., 2007).

**Clinical Aspects of Ischemic Preconditioning**

Numerous studies have been well demonstrated the clinical potential of preconditioning in patients of ischemic heart disease. Various in-vivo models of ischemic preconditioning in human myocardium have been developed, including warm-up phenomenon, preinfarction angina, angioplasty studies and other surgical studies (Yellon and Downey, 2003). The ischemic preconditioning phenomenon was well demonstrated in human atrial muscle of patients undergoing coronary artery bypass graft surgery (CABG) (Walker et al., 1994). Other in-vitro studies also indicated that δ-opoid receptor act as a trigger in human myocardium subjected to ischemic preconditioning (Bell et al., 2000). Myocardial biopsies taken after 10 min. of cross clamping exhibited significantly higher content of ATP and reduced release of troponin (Tomai et al., 1999, Ylitalo and Peuhkurinen, 2001). Pharmacological recruitment of protection using adenosine (Mentzer et al., 2003), volatile anaesthetics ie. isoflurane (Belhomme et al., 1999; Riess et al., 2004) is an other interesting
alternative to provide preconditioning mediated cardioprotection in patients undergoing CABG (Tomai et al., 1999; Ylitalo and Peuhkurinen, 2001). The Post-transluminal coronary angioplasty (PTCA) procedure involves repeated intracoronary balloon inflations with intervening periods of perfusion which was characterized by less anginal pain, less ST-segment shift, and lower mean pulmonary artery pressure, despite a reduction in cardiac vein flow and unchanged coronary wedge pressure during second balloon inflation (Yellon and Downey, 2003).

Pre-treatment with nicorandil, a Mito K$_{ATP}$ channel opener precondition the myocardium by preventing the incidence of ventricular arrhythmias and myocardial dysfunction after coronary reperfusion (Toru et al., 2001). Further, adenosine preconditioning decrease the severity of ischemia during the first balloon inflation and that was significantly improved on subsequent balloon inflations during PTCA (Leesar et al., 2003). The warm-up phenomenon improves coronary blood flow and reduced myocardium oxygen consumption during the second period of exertion (Okazaki et al., 1993; Marber et al., 1994). This endogenous adaptation has been studied during successive ergometer or walking tests and during repeated atrial and ventricular pacings (Joy et al., 1987; Ylitalo and Peuhkurinen, 2001; Ylitalo et al., 2001). Patients with pre-infarct angina were found to have smaller creatine kinase output, less arrhythmias, less stunning and heart failure and better in-hospital outcome after thrombolytic therapy than patients without pre-infarction angina (Anzai et al., 1994; Andreotti et al., 1996; Kloner et al., 1998; Skyschally et al., 2005; Yan et al., 2008). Pre-infarct angina may activate an endogenous antithrombotic or fibrinolytic mechanism which gives more time for revascularization procedures (Haider et al., 1995; Tomoda and Aoki, 1999).
The findings from many preclinical studies in which cardioprotection has been seen in healthy animal hearts might not be reproducible in the human myocardium due to several factors such as old age, presence of comorbid disease such as diabetes, hypertension, hypercholesterolemia (Ramzy et al., 2006). Moreover, the timing and duration of myocardial ischemia, use of pharmacological agents such as oral sulfonylurea drugs or cyclooxygenase 2 inhibitors and practical constraints may complicate preconditioning protocol and limit the benefits of these drugs under such clinical conditions (Schulman et al., 2001; Riess et al., 2004).

**Diabetes Mellitus and Ischemic Preconditioning**

Diabetes mellitus is a heterogenous pathological condition that is characterised by abnormalities in carbohydrate, lipid and protein metabolism, which ultimately lead to several acute and chronic complications (Mahgoub and Abd-Elfattah, 1998). Altered metabolism in diabetes makes it prone to ischemic heart disease (Lopaschuk, 2001). Cardiovascular risk events occur at an alarming rate in diabetic patients (Kannel and McGee, 1979; Jelesoff et al., 1996; Monteiro et al., 2005; Sari et al., 2008). Hyperglycemia has been reported to increase the level of circulating free fatty acids (Lopaschuk et al., 1992), impair coronary microvascular responses to ischemia (Kersten et al., 1995), reduce the availability of nitric oxide (Giugliano et al., 1997; Bohlen and Nase, 2001), alter cellular redox state (Marfella et al., 2002), overactivate sympathetic nervous system (Zaugg and Schaub, 2004) and enhances oxygen derived free radical production (Pennathur and Heinecke, 2007; Yang et al., 2009) which further aggravate the myocardial injury.

Diabetes mellitus leads to lower glycolytic rates and impaired glucose oxidation resulting in loss of ATP (Hearse et al., 1975; Lopaschuk et al., 1992; Ravingerova et
al., 2000), accumulation of long chain fatty acyl Co.A (Pieper et al., 1984), dysfunction of endothelium (Abiru et al., 1993; Bouchard and Lamontagne, 1998), increased pro-inflammatory action of platelet activating factor and altered expression of adenosine receptors (Greden et al., 2005) and impairment of mito $K_{ATP}$ channels (Kersten et al., 2001), which aggravate the ischemia reperfusion-induced myocardial injury. Further, diabetic myocardium exhibits impaired ion transport (Kusama et al., 1992; Shimoni et al., 2003) as evidenced by altered sodium homeostasis attributed to greater influx of sodium via Na$^+$/K$^+$/2Cl$^-$ co-transporter during ischemia which aggravates myocardial injury (Ramasamy et al., 2001). Rac1, a potent determinant of intracellular redox status, (Grizot et al., 2001) was noted to be impaired by ischemia in isolated diabetic rat heart. Hence, Rac1 mediated activation and expression of hypoxia inducing factor1 alpha expression (HIF1$\alpha$) also gets diminished which further accelerates the injury to myocardium (Marfella et al., 2002).

In contrast, some of the experimental studies also demonstrated that the diabetic heart is resistant to ischemia and reperfusion injury (Kusama et al., 1992; Liu et al., 1993; Tosaki et al., 1996; Kersten et al., 2000; Ferdinandy et al., 2007; Otani et al., 2007) which may be due to the depressed sarcoplasmic calcium pump activities (Ganguly et al., 1983), decrease in sensitivity of $\beta$-adrenergic stimulation (Atkins et al., 1985, Schaffer, 1999), elevated antioxidant defenses (Wohaeib and Godin, 1987; Matejikova et al., 2008), depressed sodium-calcium exchange (Allo et al., 1991), upregulation of PKC (Moon et al., 1999) and release of protective calcitonin gene related peptide (CGRP) (Lu et al., 2001). Also, pathophysiological stimuli increase oxidative stress that upregulate hemeoxygכנase-1 (HO-1), a cytoprotective heme degrading enzyme. HO-1 has a critical role in cardiac homeostasis in response to
oxidative stress-induced injury (Melo et al., 2002) and protects diabetic heart by preventing sloughing of endothelium of myocardium (Pileggi et al., 2001). These studies also demonstrated that absence of HO-1 renders animals more vulnerable to myocardial ischemia-reperfusion damage and diabetes exacerbates the injury (Liu et al., 1992). Another study focused on the haptoglobin genotype (Hp) in explaining oxidative stress in diabetic mice. The Hp1 allele is associated with antioxidant and anti-inflammatory property due to IL-10 formation on giving ischemia and reperfusion and therefore shows no further reduction of infarct size with antioxidant treatment whereas Hp2 allele increases lipid peroxidation and oxidatively active iron formation on occlusion of left anterior descending coronary artery (LAD) but limit infarction on antioxidant treatment (Blum et al., 2007). In addition, the age of the animals has also been shown to alter the susceptibility to ischemia reperfusion injury. Diabetic rat hearts were found to be resistant to ischemic insult at the age of 3 months, but were more sensitive to ischemic insult at the age of 6 months (Maddaford et al., 1997).

The accumulating evidences in the literature reveal the controversies in ischemic preconditioning mediated cardioprotection in diabetic rat heart (Paulson, 1997; Ferdinandy et al., 1998, 2007). It has been reported that hyperglycemia is the major predictor of extent of myocardial infarction which is supported by the fact that myocardial infarct size is linearly related to blood glucose concentration in canine model of diabetes subjected to ischemic preconditioning (Kersten et al., 2000). The absence of early and late ischemic preconditioning has been demonstrated against stunning in conscious diabetic sheep model. Sarcolemmal $K_{ATP}$ channel dysfunction which cause inadequate calcium handling during ischemia reperfusion, thereby
resulting in less mechanical recovery from stunning in diabetic sheep heart whereas mito K$_{ATP}$ channel did not appear to account for the above said finding (del Valle et al., 2002).

The impairment of PI-3K/AKT pathway during ischemic preconditioning in diabetic heart has been reported. Elevated preconditioning stimulus is, therefore, required to reach the threshold level of cardioprotection in diabetic myocardium (Tsang et al., 2005; Wynne et al., 2007). Incontrast, ischemic preconditioning has been reported to produces cardioprotection in diabetic myocardium (Kusama et al., 1992; Liu et al., 1993; Tosaki et al., 1996; Kersten et al., 2000; Ferdinandy et al., 2007). Preconditioning effect in diabetic rat is due to the preservation of mitochondrial ATPase and adenine nucleotide translocase activities attributed to the restoration of high energy phosphates after sustained ischemia and reperfusion. They also demonstrated reduced production of lactic acid during sustained ischemia and concomitant attenuation of intracellular acidosis (Tatsumi et al., 1998). Further, hyperglycemia may present a stressful stimulus leading to concomitant upregulation of endogenous stress protein ie. HSP-27 in diabetic mice which brought up a potential role in cardioprotection and compensate for detrimental effects of hyperglycemia in diabetes (Chen et al., 2005).

The conflicting results of various studies on ischemic preconditioning in diabetes may be due to difference in experimental conditions and model (utilizing regional flow, global, low flow ischemia or hypoxia) used, species subjected to ischemic preconditioning, duration and chronicity of diabetes, presence of superimposed comorbid conditions such as atherosclerosis, hypertension and choice of end points used (Paulson et al., 1997).
Hyperlipidaemia and Ischemic Preconditioning

Ischemic preconditioning exerts remarkable cardioprotection, in the normal heart but the cardioprotective effect of ischemic preconditioning in hyperlipidaemic heart is controversial (Ferdinandy et al., 1998, 2007).

The early cardioprotective effect of ischemic preconditioning was shown to be lost in hypercholesterolemic rabbits (Ueda et al., 1999), rats (Ferdinandy et al., 1998; Kocic et al., 1999; Kocsis et al., 2010), and also in humans (Kyriakides et al., 2002). In contrast, according to reports of other studies ischemic preconditioning produces cardioprotection in hyperlipidaemic mice (Jung et al., 2000) and rabbits (Kyriakides et al., 2002; Iliodromitis et al., 2006).

Hyperlipidaemia also influences the extent of myocardial ischemia and reperfusion – induced injury and interferes with the cardioprotective effect of ischemic preconditioning (Ferdinandy et al., 2007). Moreover, hyperlipidaemia is a well known major risk factor for ischemic heart disease (Grundy et al., 2004). The endogenous substances such as NO and heat shock protein 72 (HSP 72)-mediated second window of the cardioprotective effect of ischemic preconditioning is also abolished during hyperlipidaemia (Hoshida et al., 1996; Ferdinandy et al., 1998; Csont et al., 2002; Kyriakides et al., 2002). Moreover, the data also suggests that hyperlipidaemia produces inhibition of myocardial stress adaptation (Ferdinandy et al., 1998).

The mechanism by which hyperlipidaemia attenuates the cardioprotective effect of ischemic preconditioning is not precisely known. The accumulating data in the literature suggest that a decrease in the level of NO (Hoshida et al., 1996; Ferdinandy et al., 1998; Cohen et al., 2010) and inhibition of myocardial tetrahydrobiopterin
(BH₄) (Tang et al., 2005) are responsible for attenuation of the cardioprotective effect of ischemic preconditioning. Moreover, excessive generation of ROS like superoxide anion and peroxynitrite radical has been reported to increases oxidative stress in the myocardium which may contribute to attenuation the infarct size limiting effect of ischemic preconditioning (Szlavassy et al., 2001; Onody et al., 2003; Puskas et al., 2004; Giricz et al., 2006). In addition, enhanced activation of caspase-3 and subsequently apoptotic mediated cell death may be responsible for attenuation of the ischemic preconditioning mediated protection of cardiomyocytes in hyperlipidaemia (Wang et al., 2003). The impaired activation of mito K⁺ATP channels (Katakam et al., 2007) and a decrease in ecto-5'-nucleotidase activity may be responsible for attenuation of the cardioprotective effect of preconditioning (Ueda et al., 1999). Furthermore, hyperlipidaemia attenuate the ischemic preconditioning-induced inhibition of myocardial matrix metalloproteinase-2 (MMP-2), which is detrimental to ischemia-reperfusion induced injury (Giricz et al., 2006). Moreover, the accumulation of cholesterol in the sarcolemmal and mitochondrial membrane may be responsible for attenuation of ischemic preconditioning induced cardioprotection (Onody et al., 2003).

Numerous studies have reported that short term or acute cholesterol feeding in experimental animals may render the myocardium more susceptible to ischemia and reperfusion-induced injury (Hoshida et al., 1996; Jung et al., 2000). On the contrary, prolonged hypercholesterolemia has been documented to protect myocardium from ischemia and reperfusion-induced injury (Ferdinandy et al., 1998).
The Mitochondrial Permeability Transition Pore (MPTP)

Under normal physiological conditions, the mitochondrial inner membrane is impermeable and maintains the potential gradients across the membrane which is essential for oxidative phosphorylation. However, during the adverse condition such as; high matrix calcium, oxidative stress, high phosphate and low adenine nucleotide concentrations, a pore get formed in the inner mitochondrial membrane known as the mitochondrial permeability transition pore (MPTP) (Halestrap et al., 2002, 2004; Bernardi et al., 2006). During opening of this pore the substances of less than 1.5 kDa easily cross and disrupt the permeability barrier of the inner mitochondrial membrane. This also allows unrestricted movement of proton across the inner membrane, which leads to the uncoupled oxidative phosphorylation and prevent the synthesis of ATP and hydrolyse the stored ATP rather than synthesise it. Under such conditions, intracellular ATP concentrations rapidly decline, leading to the activation of degradative enzymes such as phospholipases, nucleases and proteases (Halestrap et al., 2004; Solaini and Harris, 2005; Halestrap, 2006). Moreover, it has been reported that opening of a single pore for a moment, activates further opening of other pores in the same mitochondrion (Solaini, and Harris, 2005) and causes extensive swelling of mitochondria, which leads to cellular apoptosis (Halestrap, 2005; Martinou, 2001; Bernardi, et al., 2006).

The molecular structure of the mitochondrial permeability transition pore remains changeable (Halestrap et al., 2002; Halestrap, 2005; Bernardi et al., 2006), but it has been well elucidated that this change in molecular conformation of this pore is triggered by calcium and is facilitated by cyclophilin D (CyP-D), a peptidyl-prolyl cis-trans isomerase (Waldmeier et al., 2003; Halestrap, 2005). Treatment with
cyclosporin A (CsA) an inhibitor of CyP-D, prevents the opening of MPTP (Kroemer et al., 1998; Woodfield et al., 1997) Also, mitochondria of CyP-D knockout mice is much less sensitive to calcium, and are inhibited by CsA (Basso et al., 2005; Nakagawa et al., 2005). Adenine nucleotide translocase (ANT) is the major membranal component where the CyP-D interacts and opens the MPTP (Halestrap, 2006). Carboxyatractyloside (CAT), inhibits the MPTP opening by trapping ANT in its “c” conformation and in contrast, another inhibitor of the ANT, bongkrekic acid, that causes the carrier to take up the alternative “m” conformation, and inhibit the MPTP opening (Halestrap, 1991). Many other proteins have been proposed to be components of the MPTP, including the peripheral benzenediazipine receptor, creatine kinase and the voltage dependent anion channel (VDAC) (Halestrap et al., 2002, 2004; Bernardi et al., 2006). Among these most active protein is VDAC, which allow the opening of MPTP by intracting with ANT and by forming a contact sites between the inner and outer mitochondrial membranes (Bernardi et al., 2006; Halestrap et al., 2002, 2004).

It has been demonstrated that MPTP remain closed under normal physiological conditions and get opened during reperfusion after sustained ischemic insult (Griffiths and Halestrap, 2005; Hausenloy et al., 2004b). It has been noted that MPTP also get opened after prolonged ischaemia (Borutaite et al., 2001; Miura and Tanno, 2010). However, reportes from other laboratories do not support the hypothesis of opening of MPTP during ischaemia (Halestrap, 1991; Griffiths and Halestrap, 1995; Kerr et al., 1999; Kim et al., 2006). During reperfusion ROS production get started (VandenHoek et al., 2000; Kevin et al., 2003) and mitochondria get energised that lead to increased
uptake of calcium into the mitochondria, and facilitates the opening of MPTP (Halestrap et al., 2004; Halestrap, 2006).

Griffiths and Halestrap (1993) have demonstrated that administration of cyclosporine A, an MPTP inhibitor, before the global ischemia protect the heart from ischemia-reperfusion induced myocardial injury. Later on in other studies it has been reported that administration of CsA analogues, and sanglifehrin A (CyP-D inhibitor) also provide cardioprotection (Griffiths and Halestrap, 1995; Di Lisa et al., 2001; Clarke et al., 2002). Other pharmacological agents i.e. propofol (Lim et al., 2005) cariporide (Mentzer et al., 2003) produce cardioprotection indirectly by inhibition of opening of MPTP (Javadov et al., 2003). Pyruvate is a free radical scavenger, and known to maintain level of ATP during ischemic insults (Mallet et al., 2000). Moreover, pyruvate, a most powerful agent for inhibiting MPTP opening, is also known to produce cardioprotection (Kerr et al., 1999; Mallet et al., 2005; Cohen et al., 2010).

Ischemic preconditioning has noted to produces cardioprotection against ischemia reperfusion induced injury by inhibiting the opening of mitochondrial permeability transition pore (Javadov et al., 2003). It has been reported that MPTP of preconditioned cardiac myocyte are resistant to opening by oxidative stress (Hausenloy et al., 2004a; Juhaszova et al., 2004; Matsumoto-Ida et al., 2006) and calcium (Javadov et al., 2003; Argaud et al., 2004; Khaliulin et al., 2004; Cohen et al., 2010). From the reports of different studies it has been suggested that short episodes of ischemia followed by reperfusion do not have any effect on MPTP during the resting state, and prevent the destructive changes of cardiomyocytes during sustained ischemia and reperfusion. Moreover thermal preconditioning, (Khaliulin et
Ischemic Preconditioning and Mitochondrial Permeability Transition Pore: The Signaling Pathways

Several signaling pathways have been noted be involved in ischemic preconditioning-induced cardioprotection, by exerting their effects on the mitochondria (Yellon and Downey, 2003; Inagaki et al., 2006). Due to their complex interactions, it is a challenging to point out the signaling cascade, by using suitable pharmacological agonists and antagonists of components of the different signalling kinase (Armstrong, 2004; Cohen et al., 2006; Gross and Gross, 2006; Inagaki et al., 2006; Hausenloy and Yellon, 2006; Miura and Tanno., 2010).

Ischemic preconditioning, is noted to produces cardioprotection through protein kinase C (PKC). Inhibition of PKC has been shown to block the IPC-induced cardioprotection (Armstrong, 2001, 2004), and administration of pharmacological agents which activate PKC, mimic the cardioprotective effect of IPC (Inagaki et al., 2006). Out of many isoforms of PKC, PKCε has been identified as an important component during cardioprotection induced by ischemic preconditioning (Armstrong, 2004; Inagaki et al., 2006). This cardioprotective effect of ischemic preconditioning is attenuated in PKCε knockout mice (Saurin et al., 2002) and the transgenic mice with cardiасpecific over-expression of PKCε are resistant to ischemia-reperfusion induced injury (Cross et al., 2001; Ping et al., 2002; Inagaki et al., 2006). Ischemic preconditioning is reported to translocate the PKCε from cytosol to the mitochondria and inhibit the opening of MPTP (Mitchell et al., 1995; Ping et al., 1997; Baines et al., 2002, 2003; Simonis et al., 2003; Miura and Tanno, 2010). Various agents get
released during the ischemic preconditioning such as noradrenaline, adenosine, bradykinin, and opioids; that activates PKC through their G protein-coupled receptors (Gross and Gross, 2006). Moreover, the production of reactive oxygen species (ROS) get enhanced during ischemic preconditioning (Baines et al., 1997; VandenHoek et al., 1998; Kevin et al., 2003) and activate PKC (Baines et al., 1997; Bouwman et al., 2004) by oxidizing their cysteine residue (Korichneva et al., 2002; Hool, 2006). Furthermore, administration of free radical scavengers is reported to attenuate the cardioprotective effect of IPC (Baines et al., 1997; Hausenloy et al., 2004b; Khaliulin et al., 2004, 2007)

Ischemic preconditioning nitric oxide (NO) is released which is known to mediate cardioprotection by activation of the cyclic GMP dependent protein kinase (PKG), and scavengers of NO and inhibitors of PKG have been noted to attenuate the cardioprotective effect of ischemic preconditioning (Costa et al., 2005; Cohen et al., 2006; Jones and Bolli, 2006; Cohen et al., 2010). It has been suggested that PKG in an upstream of PKCε which open the mitochondrial ATP-sensitive potassium (mito K_{ATP}) channel during the IPC (Costa et al., 2005). However, it has been reported that opening of the mito K_{ATP} channel leads to generation of ROS which produce cardioprotection by inhibiting PKCε mediated MPTP opening (Costa et al., 2006).

The pro-survival kinases such as phosphatidylinositol-3-phosphate kinase (PI-3-kinase)/protein kinase B (Akt) get activated during the ischemic preconditioning (Hausenloy et al., 2005; Hausenloy and Yellon, 2006; Han et al., 2010). The phosphorylation of Akt is enhanced by ischemic preconditioning and administration of inhibitors of either Akt or PI-3-kinase have been reported to limit the preconditioning induced cardioprotection (Hausenloy et al., 2004, 2005; Tsang et al.,
Activation of PI-3K/Akt is noted to enhance the generation of NO by activating endothelial nitric oxide synthase (eNOS) (Cohen et al., 2006; Hausenloy and Yellon, 2006). Juhaszova et al., (2004) suggested that all these kinases produce cardioprotection by phosphorylating and inhibiting the glycogen synthase kinase-3β (GSK-3β). GSK3β is a pro-apoptotic kinase which is constitutively active and that gets inhibited by its phosphorylation and the resultant inhibition of its apoptotic activity stimulates cell survival (Jope and Johnson, 2004). Inhibition of the GSK-3β is noted to limit the apoptotic mediated cell death by inhibiting the opening of MPTP (Juhaszova et al., 2004; Pastorino et al., 2005; Miura and Tanno, 2010).

The brief episodes of ischemic insults are reported to increase the cytosolic level of AMP by depleting ATP, which leads to the activation of AMP-activated protein kinase (AMPK) (Young et al., 2005). During pharmacological preconditioning the activity of AMPK gets elevated in the cardiomyocytes (Young et al., 2005). Moreover, it has been reported that the cardioprotective effect of IPC is abolished in AMPK knock out mice and their heart is more susceptible to ischemia-reperfusion-induced injury (Russell et al., 2004). AMPK is downstream of PKC and administration of PKC inhibitor is reported to attenuate the ischemic preconditioning induced activation of AMPK in the myocardium (Nishino et al., 2004) However, according to the report from an other laboratory, IPC induced cardioprotection remained intact in the presence of AMPK inhibitor (Khaliulin et al., 2007).

It is possible to say that the cardioprotective signals initiated by brief episodes of stimuli that induced ischemic preconditioning may be interacting together or may run parallel and ultimately produce cardioprotection, by inhibiting the opening of MPTP.
Molecular Basis of Inhibition of The MPTP by Ischemic Preconditioning

The sensitivity of MPTP of isolated mitochondria is reduced in the presence of phorbol ester (Baines et al., 2003), ATP (Halestrap et al., 2002, 2004) and cGMP analogue (Kim et al., 2004). IPC is noted to reduce the sensitivity of calcium-induced opening of MPTP during ischemia and reperfusion by phosphorylating the component of MPTP (Javadov et al., 2003; Khaliulin et al., 2004). Also, it has been reported that, sensitivity of the calcium-induced opening of MPTP get reduced, under conditions that elevate cytosolic cyclic AMP levels (Hughes and Barritt, 1978; Prpic et al., 1978; Armston et al., 1982). In addition, the cytosolic kinases i.e. PKCε and GSK3β which get phosphorylated by IPC may enter into mitochondrial matrix by crossing its inner membrane and regulate the opening of MPTP (Ping et al., 1997; Baines et al., 2002; Juhaszova et al., 2004; Miura and Tanno, 2010).

The mitochondrial calcium and reactive oxygen species are the major factors which regulate the opening of MPTP (Juhaszova et al. 2004; Cohen et al., 2010) and the level of Ca^{2+} (Miyata et al., 1992; Griffiths et al., 1998) and ROS (Kim et al., 2006) are increased during the ischemia followed by reperfusion. The IPC is noted to reduce ROS production after sustained ischaemia and during reperfusion (VandenHoek et al., 2000; Narayan et al., 2001; Ozcan et al., 2002; Kevin et al., 2003) and also to decrease mitochondrial calcium overload (Ylitalo et al., 2000; Murata et al., 2001; Schulz et al., 2001; Wang et al., 2001). Therefore, it have been postulated that the stimuli of IPC may also regulate the opening of MPTP, by decreasing the noxious stimuli i.e. Ca^{2+} and ROS. It has been shown that IPC failed to decrease the sensitivity of calcium-induced MPTP opening in isolated mitochondria (Javadov et al., 2003; Khaliulin et al., 2004) and that the mitochondria isolated from preconditioned hearts.
were less sensitive to MPTP opening than were mitochondria isolated from control hearts (Argaud et al., 2004; Khaliulin et al., 2004; Khaliulin et al., 2007).

It has been reported that opening of ATP-sensitive potassium channels (K<sub>ATP</sub>) are responsible for IPC-induced cardioprotection and that administration of glibenclamide, a K<sub>ATP</sub> channel blocker, abolished the cardioprotective effect of IPC (Hanley and Daut, 2005; Kane et al., 2005). The IPC mediated cardioprotection and of pharmacological preconditioning is attenuated in heart obtained from K<sub>ATP</sub> channel knock out rat and these rats are more sensitive to ischaemia/reperfusion induced injury (Suzuki et al., 2002, 2003). Thus, opening of K<sub>ATP</sub> channels produces cardioprotection, i) by improving the production of ATP (Rourke, 2000), ii) by maintaining the mitochondrial matrix volume (Garlid et al., 2003) and iii) by inhibiting the opening of MPTP (Holmuhamedov et al., 1999; Wang et al., 2001; Oldenburg et al., 2003; Rourke, 2004; Sato et al., 2005).

Connexion 43 (Cx43) is the major protein of the gap junctions which connect adjacent cardiomyocytes and propagate the action potential, and other signalling molecules and metabolites from cell to cell (Schulz and Heusch, 2004, 2006; Dhein, 2005). Recently, it has been proposed that IPC-induced cardioprotective response of mitochondria of cardiomyocytes is mediated through Cx43 (Boengler et al., 2005; Halestrap, 2006; Rodriguez-Sinovas et al., 2006; Schulz and Heusch, 2006). Cx43 is normally only partially phosphorylated with low conductance, but progressive dephosphorylation occurs during ischemia causing increased conductance of death signaling and propagating injury from one cell to another cell (Dhein, 2005). IPC is noted to produce cardioprotection by preventing the progressive dephosphorylation of Cx43 during ischemia (Jain et al., 2003; Schulz et al., 2003). Also the cardioprotective
effect of IPC is attenuated in heart from Cx43-deficient mice (Schulz et al., 2003). Cx43 is noted to produces cardioprotection by inhibiting the opening of MPTP during IPC (Boengler et al., 2005; Halestrap, 2006; Rodriguez-Sinovas et al., 2006). Moreover it has been reported that Cx43 get translocated into mitochondria during ischemic preconditioning (Halestrap, 2006; Rodriguez-Sinovas et al., 2006). However, Hausenloy and colleagues have suggested that transient opening of the MPTP is essential for cardioprotective effect of IPC and that this IPC mediated cardioprotection is abolished in the presence of MPTP inhibitor (Hausenloy et al., 2004b).

3.9 Glycogen Synthase Kinase -3 Beta (GSK-3β)

In 1984 it was found that a new enzyme i.e. glycogen synthase kinase (GSK-3) is involved in the regulation of the synthesis of glycogen (Woodgett, 1991). GSK-3 is of two isoforms GSK-3α and GSK-3β, there is 86% structural homology and 97% similar kinase activity (Woodgett, 1990, 1991). Both kinases have an inhibitory Ser phosphorylation site at their N-termini (S21 for α and S9 for β) and a facilitative Tyr site in their catalytic loop (Y279 for α and Y216 for β) (Stambolic and Woodgett 1994; Cross et al., 1995). With regard to the Tyr site, while its mutation to Ala impairs the full activation of GSK-3 kinase activity, the regulation of its phosphorylation is not well understood (Hughes et al., 1993; Plyte et al., 1996). It has been suggested that phosphorylation of GSK-3 at Tyr site is constitutive. Moreover GSK-3 has several unique features compared with other kinases: (a) In basal state GSK-3 is in active state and extracellular stimulation inhibits GSK-3 activity, while other kinases are activated by ligands; (b) phosphorylation at Ser21 for GSK3-α and Ser9 for GSK-3β inhibits GSK-3 kinase, while most other kinases are activated by
phosphorylation; (c) in general, GSK-3β requires ‘primed’ phosphorylation of its substrates by other kinases before GSK-3β can phosphorylate them, except for some cases including β-catenin and Axin; (d) GSK-3β mainly plays a role as a negative regulator in signaling processes, except in the NFkB signaling pathway.

GSK-3β regulates the blood glucose level by the enzyme glycogen synthase (GS) which stores glucose in the form of glycogen (Ferrer et al., 2003). During basal conditions, GSK3α and β are in active state and phosphorylate it downstream substrate i.e. glycogen synthase and inhibits its activity, subsequently inhibits the synthesis of glycogen. Insulin is noted to activate its signaling cascade; insulin receptor (IR)-insulin receptor substrate (IRS)/PI-3K/Akt leads to the phosphorylation of GSK3 at the regulatory Ser21 at alpha or 9 residues at beta and GSK3 kinase activity is inhibited. This activates glycogen synthase and initiates the process of synthesis of glycogen (Woodgett, 1991; Eldar-Finkelman, 2002). In addition, GSK-3β regulates protein synthesis by controlling the activity of initiation factor 2B (eIF2B) in the same manner as glycogen synthase (Welsh and Proud 1993; Welsh et al., 1997). During basal conditions, GSK-3β phosphorylates and inactivates eIF2B, but upon insulin stimulation, the inhibition of GSK-3β induces the dephosphorylation and activation of eIF2B (Cross et al., 1994; Armstrong et al., 2001).

The activity of GSK-3β is regulated by phosphatidylinositol 3-kinase (PI-3K)/Akt, mitogen-activated protein kinase-activated protein kinase-1 (MAPKAP-K1), a protein kinase downstream of the mitogen-activated protein kinase (MAPK) cascade, and p70 ribosomal S6 kinase-1 (Cross et al., 1995; Cohen and Frame, 2001). The GSK-3β play an important role in the pathophysiology of various disorders i.e. alzheimer, memory reconsolidation, uncontrolled tissue growth, i.e. cancer, bipolar mood...
disorder and in ischemia-reperfusion injury (Cohen and Frame, 2001; Liao et al., 2003).

**GSK-3β and Cardioprotection**

Juhaszova et al., (2004) reported that the opening of MPTP of cardiomyocytes is regulated by GSK-3β. They demonstrated that the threshold for ROS-induced opening of MPTP is elevated by phosphorylation and subsequent inhibition of GSK-3β. Moreover, it has been reported that in genetically knockout GSK-3β animals, MPTP gets easily opened in response to ROS and Ca²⁺(Gomez et al., 2008). Ischemic postconditioning is noted to significantly elevate the threshold of MPTP opening in cardiac mitochondria. How the phosphorylation and subsequent inactivation of GSK-3β increases the threshold for MPTP opening remains unclear? It has been reported that, hexokinase-II (HK-II) is preserved in the MPTP complex by inhibition of GSK-3β (Miyamoto et al., 2005; Pastorino et al., 2005). The activation of GSK-3β induces release of HK-II from the mitochondria via phosphorylation of voltage dependant anion channel (VDAC), which enhances susceptibility of the cells to necrosis by opening of MPTP (Pastorino et al., 2005). It has been suggested that regulation of opening of MPTP by GSK-3β i.e. binding of phospho-GSK-3β to ANT suppresses interaction of ANT with CypD, a trigger of MPTP opening (Nishihara et al., 2007). In isolated rat hearts, reperfusion after sustained ischemia, induced translocation of cytosolic GSK-3β to the mitochondria, where it formed a complex with adenine nucleotide translocase (ANT) and VDAC. Phosphorylation of GSK-3β by ischemic preconditioning and erythropoietin (EPO) receptor activation is dependent on protein kinase C (PKC) and phosphatidylinositol 3-phosphate kinase (PI-3K), and phospho-GSK-3β binds to ANT. Interestingly, this phospho-GSK-3β-ANT interaction was
associated with reduction of CypD-ANT binding and inhibiting the opening of MPTP. Also, p53-mediated regulation of MPTP might be suppressed by inhibition of GSK-3β activity. Phosphorylation of p53 by GSK-3β enhances its functional activity and its translocation to the nucleus and mitochondria (Watcharasit et al., 2003; Han et al., 2010). Venkatapuram et al., (2006) showed that an inhibitor of p53, pifithrin-α, sensitized the myocardium to isoflurane-induced protection, mediated through phospho-GSK-3β-mediated. This beneficial effect of pifithrin-α was abolished by an MPTP opener, atractyloside, indicating link of the p53 with MPTP. Inhibition of GSK-3β suppressed ATP hydrolysis by reducing ATP transport from the cytosol to the mitochondria (Das and Kukreja, 2008). Suppression of ATP hydrolysis during ischemia would prevent both ATP depletion and accumulation of inorganic phosphate which promotes MPTP opening. All of the mechanisms discussed here are not mutually exclusive and might contribute in concert to MPTP inhibition.

**GSK-3β and Tolerance of Cardiomyocytes to Apoptosis and Necrosis**

It has been documented that GSK-3β has two opposite roles in apoptotic death of cardiac and non-cardiac cells depending on the trigger of apoptosis. GSK-3β activity limits pro-apoptotic signaling from death receptors i.e. TNF-R1, Fas, DR4 and DR5 (Schwabe and Brenner, 2002; Liao et al., 2003; Song et al., 2004; Rottmann et al., 2005). Inhibition of GSK-3β in the cells with activated death receptors enhanced activation of caspases-8 and -3, Bid cleavage and apoptosis, indicating that GSK-3β suppresses pro-apoptotic signals upstream of caspase activation (Schwabe and Brenner 2002; Liao et al., 2003; Song et al., 2004). Thus, inactivation of GSK-3β under the condition of stimulation of death receptors promotes apoptosis. In contrast, apoptosis induced by intrinsic mechanisms is facilitated by active GSK-3β that it’s
non phosphorylated forms. Pro-apoptotic functions of GSK-3β have been shown in apoptosis by withdrawal of growth factors (Cross et al., 2001; Linseman et al., 2004, Sinha et al., 2005), DNA damage (Watcharasit et al., 2003; Tan et al. 2006), mitochondrial toxins (King et al., 2001), ischemia (Brywe et al., 2005; Kaga et al., 2005), oxidative stress (King and Jope, 2005) and other conditions that trigger apoptosis via the mitochondrial pathway.

Phosphorylation of the GSK-3β substrates i.e. p53, heat shock factor-1, myeloid cell leukemia sequence-1, Bax is involved in the pro-apoptotic functions. Phosphorylation of p53 in the nucleus leads to p53-mediated transcription of pro-apoptotic genes (Watcharasit et al., 2003; Beurel et al., 2004) phosphorylation of HSF-1 inhibits its function as a survival-promoting transcription factor (Bjur and Jope, 2000; Khaleque et al., 2005), phosphorylation of MCL-1 induces ubiquitinylation and subsequent degradation of this anti-apoptotic Bcl-2 protein (Maurer et al., 2006; Zhao et al., 2007) and phosphorylation of Bax promotes its localization in the mitochondria and apoptosis (Linseman et al., 2004). The share of these GSK-3β substrates to apoptosis seems to be different, depending on cell types, experimental conditions and triggers of apoptosis. Although the role of GSK-3β in apoptosis of cardiomyocytes has not been fully elucidated, evidence to date supports its significant contribution to apoptosis induced by ischemia/reperfusion (Yin et al., 2004, 2005; Das et al., 2008; Gao et al., 2008; Vigneron et al., 2011), hypoxia/re-oxygenation (Bergmann et al., 2004), β-adrenoceptor activation (Menon et al., 2007) and during pressure overload (Hirotani et al., 2007). Apoptosis by these insults was suppressed by overexpression of the adrenomedullin gene (Yin et al., 2004) or kallikrein gene (Yin et al., 2005) or treatment with statins (Bergmann et al., 2004), all of which induced phosphorylation.
of GSK-3β. Furthermore, this protection from apoptosis was inhibited by negative Akt or active GSK-3β mutant and mimicked by pharmacological inhibitors of GSK-3β. (Bergmann et al., 2004; Yin et al., 2004, 2005; Menon et al., 2007; Das et al., 2008; Gao et al., 2008; Vigneron et al., 2011). Transgenic mice that cardio-specifically express dominant negative GSK-3β showed significantly fewer apoptotic cardiomyocytes after pressure overloading by aortic constriction (Hirotani et al., 2007).

These findings indicate that GSK-3β activity determines the fate of cardiomyocytes exposed to apoptosis inducers. However, the intracellular localization of phospho-GSK-3β responsible for the anti-apoptotic function and the mechanism downstream of GSK-3β phosphorylation in cardiomyocytes remain unclear. Recently, it has been noted that the translocation of phosphorylated GSK-3β to the mitochondria is important for inhibition of oxidative stress-induced apoptosis of cardiomyocytes (Ohori et al., 2008). Moreover, it has been noted that the phosphorylation of GSK-3β, pre-existing in the mitochondria by Akt, affords protection from oxidant stress-induced apoptosis, possibly by suppressing Bax translocation, in cardiomyocytes. Phosphorylation of GSK-3β in the mitochondria is possibly achieved by translocation of Akt to the mitochondria (Miyamoto et al., 2005; Kobayashi et al., 2008; Ohori et al., 2008; Miura and Tanno, 2010).

The inhibition of GSK-3β produces cardioprotection against ischemia-reperfusion-induced injury through inhibition of opening of mitochondrial permeability transition pore during the reperfusion phase (Juhaszova et al., 2004; Feng et al., 2005; Vigneron et al., 2011). Glycogen synthase kinase-3β activity is elevated during diabetes mellitus (Eldar-Finkelman et al., 1999; Henriksen et al., 2003), may cause glucose
intolerance (Pearce et al., 2004). Inhibition of GSK-3β may improve glucose tolerance in diabetes mellitus (Cline et al., 2002). Diabetes mellitus activates, GSK-3β perhaps by impairing its upstream pathways (Gross et al., 2007).

The transgenic mice that overexpress GSK-3β are hyperlipidaemic (Pearce et al., 2004). Moreover, hyperlipidaemia is reported to increase the activity of PPAR-α (Kewalramani et al., 2006) which is also known to activate GSK-3β by inhibiting its phosphorylation (Li et al., 2007). Furthermore it has been documented that hyperlipidaemia may activate GSK-3β through activation of platelet activating factor (PAF) (Prescott et al., 1996; Tong et al., 2001). Therefore the involvement of GSK-3β, in the reduced effect of infarct size limiting potential of ischemic preconditioning needs to be evaluated.

It has been suggested that anti-infarct tolerance of heart is increased by ischemic preconditioning (Tong et al., 2004; Budas et al., 2006; Nishihara et al., 2006) and ischemic postconditioning (Gomez et al., 2008; Yin et al., 2009). GSK-3β is a converging point of multiple protective signaling pathways during ischemic preconditioning i.e. pro-survival protein kinases; including Akt, PKC-ε, extracellular signal-regulated kinase (ERK) and protein kinase G, phosphorylation by these ultimately inhibit the GSK-3β. GSK-3β inhibitors SB-216763 or lithium chloride administered before either ischemia or reperfusion has been noted to decrease the infarct size (Tong et al., 2004; Nishihara et al., 2006; Gross et al., 2006; Obame et al., 2008; Mozaffari and Schaffer, 2008; Cohen et al., 2010). It is interesting to note that inhibitors of GSK-3β increase the level of phospho-GSK-3β, in cardiomyocytes (Zhang et al., 2003). Moreover, the level of phospho-GSK-3β is increases during ischemic preconditioning or administration of erythropoietin, or combination of these
two, and by administration of inhibitors of PKC or PI-3K together with IPC-EPO combination before the global ischemia and ischemia reperfusion. Levels of phospho-GSK-3β tightly correlated with infarct sizes (Nishihara et al., 2006). This relationship between phospho-GSK-3β and infarct size is unlikely to be nonspecific, because there was no correlation between infarct size with level of phosphor-Akt or phospho-STAT3 on reperfusion.

The role of phospho-GSK-3β in the cardioprotective effect of ischemic preconditioning is variable in species of the animals used in experimental studies. The protective effects of ischemic preconditioning and postconditioning were not lost in GSK-3β knock-in mice in which Ser 9 of GSK-3β were changed to Ala (Nishino et al., 2008). However, Gomez et al., (2008) reported that the infarct size-limiting effect of postconditioning was lost in GSK-3β knock out mice. In addition, Skyschally et al., (2009) recently reported that the infarct size-limiting effect of ischemic postconditioning was maintained in pigs in which phosphorylation of Akt, ERK and GSK-3β was inhibited by inhibitors of PI-3K and ERK. These conflicting finding suggest that role of phospho-GSK-3β as a determinant of myocyte tolerance to reperfusion-induced necrosis may be species dependent.

**Heat Shock Proteins (HSP)**

The term "heat shock protein" (HSP) are the group of proteins differentiated by molecular weight (i.e., HSP 60, HSP 72, and HSP 90 families). Within families of HSP there are subsets; constitutively expressed proteins referred to as heat shock cognates (HSCs), whereas proteins expressed largely under conditions of stress are referred to as the HSPs. (Amrani et al., 1996; Kumar et al., 2011). The cells exposed to elevated temperature, ethanol, heavy metals, or other noxious stimuli show
increased expression of HSPs. HSCs and constitutively expressed HSPs prevent misfolding or aggregation of newly synthesized polypeptides, allowing polypeptides to traverse biomembranes and promoting proper folding and oligomerization of newly synthesized proteins under basal level (Amrani et al., 1996; Serrano et al., 2011) and has been referred to as molecular chaperones.

The physiological role of the molecular chaperones is protection of other proteins against aggregation, folding of nascent proteins or refolding of damaged proteins, degradation of severely damaged proteins. Chaperones are, highly conserved proteins that utilize ATP for conformational changes to refold other proteins, and also regulate the activity of other enzymes (Hartl, 1992; Thirumalai & Lorimer, 2001; Serrano et al., 2011). The cytosolic expression of HSP’s is increased during stress and due to this phenomenon, HSP’s are often called stress proteins. During cellular stress the functional activity of chaperone of HSP’s get elevated this triggers their induction and leads to a ‘cascading amplification’ of the activity of other available chaperone. The synthesis of HSP is induced by the activation of the heat shock factor (HSF)-1. In resting cells chaperones bind to HSF-1 and keep it in an inactive state. During stress, these inhibitory chaperones target to other proteins, this leads to the dissociation of the chaperone/HSF-1 complex, resulting in translocation of transcription factor into the nucleus. HSP’s make a complex with each other and recruit various others smaller proteins, called cochaperones and the central chaperone complex of the cytoplasm is put together around HSP 90 and is called foldosome (Pratt & Toft, 2003; Yin et al., 2009). HSP 90 is an ATP-dependent chaperone and is responsible for the folding of more than 200 proteins of various signaling pathways, and also in the refolding of
many denatured proteins after cellular stress (Csermely et al., 1998; Pratt & Toft, 2003; Serrano et al., 2011).

HSP may regulate the activity of various kinases such as tyrosine kinases, Raf, Akt and cyclin-dependent kinases. It has been documented that HSP is necessary for the maturation of several transcription factors, like the nuclear hormone receptors and the hypoxia-inducible factor-1 and regulate the activity of nitric oxide synthases and other antiapoptotic proteins (Csermely et al., 1998; Buchner, 1999; Pratt & Toft, 2003; Zhao et al., 2005; Serrano et al., 2011). Pharmacological inhibition of HSP 90 leads to impairment of the number of proliferative signals, including the Akt dependent survival pathway (Munster et al., 2001, 2002; Basso et al., 2002) and sensitize the lysis of tumor cells under stress conditions (Sreedhar et al., 2004).

However, it has been documented that administration of HSP 90 inhibitors, increases the expression of as HSP by transcriptional activation of HSF-1 by disrupting of HSP/HSF-1 complexes (Morimoto, 2002; Yin et al., 2009). Moreover, geldanamycin, a specific inhibitor of HSP 90, has been shown to activate HSF-1 (Kim et al., 1999; Bagatell et al., 2000; Yin et al., 2009) and the expression of HSP 40, HSP 72 and HSP 90 (Sittler et al., 2001).

**Heat Shock Proteins in the Cardiovascular System**

According the report of World Health Organization, the acute and chronic ischemic heart disease will be one of the major causes of death till 2020 (Murry and Lozep, 1997). The heart could benefit from protective measures from an endogenous source and upregulation of the expression of HSP’s is noted to improve tolerance against ischemia-reperfusion induced injury. Heat shock proteins can be induced by specific
stressors. Moreover, HSP 27, HSP 72, and HSP 84 are constitutively expressed in the myocardium (Manzerra et al., 1997). Under stress condition the expression of HSP 72 is upregulated in cardiomyocytes (Lutsch et al., 1997; Manzerra et al., 1997; Tranter et al., 2011). In human myocardium increased hemodynamic loading through aorta banding (Delcayer et al., 1998; Knowlton et al., 1991; Snoeckx et al., 1991; Xu et al., 1995) and injection of catecholamines, vasopressin, or angiotensin II (Moalic et al., 1989; Kohane et al., 1990) were found to stimulate HSP synthesis. Mcgrath and Locke (1995) determined HSP 72 levels in atrial biopsies of patients undergoing cardiopulmonary bypass surgery. Heat-shock is reported to significantly increase the HSP 72 content in other tissues i.e. brain, colon, liver, kidney, and spleen (Beck et al., 1995; Tranter et al., 2011). Moreover, within minutes of the stress the HSP 72 mRNA appears in the cytoplasm (Currie and Tanguay, 1991). Besides the heat stress, synthesis of HSP may be induced by other factors i.e. hormones (Moalic et al., 1989; Xu et al., 1995), reactive oxygen species (ROS) (Lu et al., 1993), and sodium arsenite (Wang et al., 1995). Nitric oxide (NO) is reported to induce the HSP 72 synthesis and administration of N-nitro-L-arginine (L-NNA), an inhibitor of NO synthase (NOS), inhibits the expression of HSP 72 (Malyshev et al., 1995; Biermann et al., 2010).

In the myocardium, the two isozymes of HSP32, i.e., heme-oxygenase-1 (HO-1) and HO-2 are constitutively synthesized (Abraham et al., 1987; Ewing et al., 1994). HO-1 is involved in the degradation of heme to biliverdin, iron, and carbon monoxide and is upregulated during hypoxia, heat stress and severe physical stress (Essig et al., 1997) by hemin, hydrogen peroxide (H$_2$O$_2$), heavy metals (Cruse et al., 1988; Ewing et al., 1994) and postischemic myocardial reperfusion (Maulik et al., 1995).

Protection of the Heart by HSP’s
Currie et al. (1988) for the first time reported that heat shock in rats improved the recovery of the cardiac function after 24 h of ischemia. Later on, a number of remarkable findings were reported: the early postischemic recovery of left ventricular contractility is significantly improved (Locke et al., 1995). It has been reported that the decrease cardiac content of HSP 72 may decrease the ischemic tolerance (Karmazyn et al., 1990; Yamashita et al., 1997; Tranter et al., 2011). Moreover, myocardium of transgenic rat shows more tolerances against ischemic injury (Hutter et al., 1994; Marber et al., 1994; Yin et al., 2009). The rabbit heart coronary flow is improved when the animals are heat-pretreated 24 h earlier (Yellon et al., 1992). Furthermore, the pig heart develops tolerance against ischemia, improve post ischemic recovery, decrease creatine kinase level and an increase in the activity of catalase and superoxide dismutase (Maulik et al., 1994, 1995). An ischemic insult was better tolerated 24 h after administration of norepinephrine due to increase in the HSP 72 level and improvement of postischemic functional recovery.

Brief ischemic episodes have been noted to trigger the synthesis of HSP 72 and HSP60 that increase the resistance against myocardial infarction after the 24 h (Marber et al., 1993). Xanthine oxidase, precursor of reactive oxygen species, also enhance the synthesis of HSP 72 and produces cardioprotection and this cardioprotective effect is significantly attenuated by concomitant administration of superoxide dismutase (Kukreja et al., 1994; Tranter et al., 2011).

Heat shock also has a beneficial effect on myocardial stunning produced after a short ischemic insult. (Marban, 1991; Robinson et al., 1995). In mice hearts with genetically induced high HSP 72 levels, the ischemic insult induced recovery is faster than control (Trost et al., 1998). It has also been reported that HSP produces
beneficial effects during ischemia by decreasing the oxidative stress on myocardium (Currie et al., 1988, Mocanu et al., 1993; Yin et al., 2009). Pretreatment with heat stress is reported that improves functional recovery following the cardioplegic arrest noted in terms of development of left ventricular pressure, contractility, and segmental shortening upon reperfusion by decreasing the release of creatine kinase, and by activating of superoxide dismutase (Liu et al., 1992). Moreover, heat pretreated hearts shows better preservation of postischemic mitochondrial ultrastructure (Currie et al., 1988), decreased depletion of ATP decreased accumulation of lactate (Wang et al., 1996) and decreased calcium accumulation into the myocardium (Currie et al., 1988; Yellon et al., 1992).

Enhanced tissue levels of HSP 72 have been reported to inhibit the myocardial apoptosis (Wong et al., 1996; Tranter et al., 2011). This is probably due to an interaction of HSP 72 at the level of the SAPK/JNK signaling pathway (Holmberg et al., 1997) and to the blocking of the conversion of pro-caspase-3 to active caspase 3 (Mosser et al., 1997). HSP is also noted to produce cardioprotection in aged animals (Snoeckx et al., 1993; Tani et al., 1997), against cardiac hypertrophy (Levy et al., 1990, Snoeckx et al., 1990, 2001) and also improve the functional recovery of transplanted myocardium (Zhang et al., 1996; Mehta et al., 1997).

HSPs and Ischemic Preconditioning

Ischemic preconditioning is an endogenous protective mechanism and is a biphasic phenomenon appears immediately just after ischemic insults and lasting up to 2-3 h, which reappears 24 h latter and termed as second window of protection (Murry et al., 1986; Baxter and Yellon 1999). Unlike ischemic preconditioning, HSP-mediated cardioprotection does not occur before 24 h after pretreatment. Brief ischemic
episodes have been reported to increases the synthesis of new HSP’s (Knowlton et al., 1991; Sharma et al., 1994).

The involvement of HSP’s in early phase of ischemic preconditioning is yet unclear. It has been noted that the HSPs mediated the early phase of cardioprotection induced by ischemic preconditioning (Eaton et al., 2000). The cytoplasmic changes during the first 24 h after the early phase of ischemic preconditioning stimulate the transcriptional activity, of HSP 72-gene (Das et al., 1992; Heads et al., 1995; Yin et al., 2009) leading to concomitant HSF1 activation and enhanced the synthesis of HSP 72 (Tanaka et al., 1998, Nishizawa et al., 1999 Zapletal et al., 2010). This hypothesis is further supported by the finding that the activity of SOD changes in a biphasic pattern, completely parallel to the cardioprotection profile (Das et al., 1992; Hoshida et al., 1993).

The prolonged duration of protection makes the second window of protection particularly relevant for its application under clinical conditions. Delayed cardioprotection after ischemic preconditioning was first reported in 1993 (Kuzuya et al., 1993; Marber et al., 1993) and these findings were confirmed by Yang et al., (1996; Zapletal et al., 2010). The improved ischemic tolerance is characterized by increased HSP 72 tissue levels and a decrease in the occurrence of arrhythmias (Vegh et al., 1991; Yin et al., 2009) ventricular fibrillation and decreased myocardial stunning (Yang et al., 1996). Thus, it may be suggested that ischemic preconditioning induced late phase of cardioprotection is due to enhanced HSP 72 levels and therefore, it should be considered as a mechanism of heat shock-mediated cardioprotection.
Heat Shock Proteins During Diabetes Mellitus and Hyperlipidemia:

The heat shock response potential is diminished during diabetes mellitus (Bruce et al., 2003), and hyperlipidemia (Csont et al., 2002). Interestingly the cardioprotective effect of ischemic preconditioning is attenuated during diabetes mellitus (Tosaki et al., 1996; Kersten et al., 2000; Ravingerova et al., 2000; Nieszner et al., 2002; del Valle et al., 2002) and hyperlipidaemia (Kocic et al., 1999; Udeda et al., 1999; Kyriakides et al., 2002; Ungi et al., 2005; Giricz et al., 2006).

The role of HSP’s in the mechanism of preconditioning has been extensively reviewed (Latchman, 2001; Snoeckx et al., 2001). It has been shown that the cardioprotective effect of preconditioning is linked to the functions of HSP 72. Heat stress or HSP 72 gene transfection into rat hearts has been shown to protect the ischemic myocardium (Arnaud et al., 2001; Jayakumar et al., 2001; Tranter et al., 2011). Therefore, the loss of the protective effect of preconditioning in these disease states is related ‘at least in part’ to diminished heat stress response. It has been documented that hyperlipidemia alters the expression of several proteins in the rat myocardium including upregulation of metallothionein and glutathione-S-transferase, as well as downregulation of proteasome component C 9, ubiquitin-like protein FUBI, HSP105, calreticulin and chaperonin subunit 5 epsilon (Puskas et al., 2004). The precise mechanisms by which diabetes mellitus and hyperlipidaemia lead to diminished heat stress response are not known. Moreover, altered membrane lipid composition and physical state (fluidity) of membranes are decisive factors in the processes of perception and transduction of stress into a signal that triggers the transcriptional activation of stress protein genes.
Nevertheless, HSP inducers and coinducers may have great therapeutic potential in diabetes mellitus and hyperlipidemia to regain the endogenous adaptation of the heart to ischemic stress. It has been noted that the reduced HSF-1 and HSP levels during diabetes are the result of reduced membrane fluidity (Hooper & Hooper, 2005; Yin et al., 2009). Moreover, diabetes mellitus leads to glycation, oxidative stress and insulin deficiency, which is associated with less fluid membranes in human mononuclear leucocytes and platelets (Tong et al., 1995; Srivastava, 2002). Heat treatment, itself causes membrane hyperfluidization (Hooper, 1999). The activity of phospholipase A2 is influenced by fluidity of membrane, and is stimulated by heat shock, resulting in release of arachidonic acid (Honger et al., 1996; Samples et al., 1999). Arachidonic acid stimulates HSF–DNA binding, increases phosphorylation of HSF-1 and upregulates translation of the HSP 72 (Jurivich et al., 1992; Yin et al., 2009; Zapletal et al., 2010). Elevated activity of membrane-bound phospholipases is associated with activation of protein kinase C (PKC), which is responsible for the phosphorylation of HSFs (Holmberg et al., 1997). However, overexpression of inducible HSP 72 is noted to downregulate the protein kinase A, PKC, and the MAP kinase, c-Jun N-terminal kinase and p38 stress-activated protein kinase (p38 SAPK) (Kiang et al., 2002; Zapletal et al., 2010). HSF-1 appears to have several additional layers of regulation in the cell, including Ras/ERK1/GSK3 pathway (Hamaguchi et al., 2003; Wang et al., 2003; Yin et al., 2009), activation of PI-3K/Akt and subsequent inhibition of GSK-3β (Bijur & Jope, 2000), small G-protein signalling such as Ras (Engelberg et al., 1994) and oxidative stress-induced membrane translocation of Rac1 (Xu et al., 2000). Simvastatin, a lipid lowering drug, blocked the oxidative stress-induced membrane translocation of Rac1, which transmit the heat stress signal from membranes to DNA to induce expression of HSP’s (Negre-Aminou et al., 2002).
However, a lipid-selective association of HSP’s with membranes, leading to increased molecular order, may in turn lead to downregulation of the heat shock gene expression (Torok et al., 1997; Tsvetkova et al., 2002).

In short, the above mentioned review of literature indicates that, glycogen synthase kinase-3β activity is elevated during diabetes mellitus (Eldar-Finkelman et al., 1999; Henriksen et al., 2003) and this may be the cause of glucose intolerance (Pearce et al., 2004) since inhibition of GSK-3β improves glucose tolerance in diabetes mellitus (Cline et al., 2002). It has been reported that diabetes mellitus may activate GSK-3β perhaps by impairing its upstream pathways (Gross et al., 2007). The transgenic mice that overexpress GSK-3β, are hyperlipidaemic (Pearce et al. 2004). Moreover, hyperlipidaemia is reported to increase the activity of PPAR-α (Kewalramani et al., 2006), which is also known to activate GSK-3β by inhibiting its phosphorylation (Li et al., 2007). Furthermore, it has been documented that hyperlipidaemia may activate GSK-3β through activation of platelet activating factor (PAF) (Prescott et al., 1996; Tong et al., 2001).

Heat shock protein 72 (HSP 72) is noted to produce late phase of cardioprotection by ischemic preconditioning (Hutter et al., 1994; Williams and Benjamin, 2000; Latchman, 2001; Snoeckx et al., 2001; Zapletal et al., 2010). The expression of HSP 72 is diminished in diabetes mellitus (Bruce et al., 2003), hyperlipidaemia (Csont et al., 2002) and condition leading to overexpression of GSK-3β (Chu et al., 1998). However pharmacological inhibition of GSK-3β is noted to increase the expression of HSP 72 (Bijur & Jope 2000; Wang et al., 2003). Thus it may be worth while to also investigate the role of GSK-3β in the modulation of the cardioprotective effect of both early and late phase of preconditioning in diabetic and hyperlipidaemic hearts.