CHAPTER 6

Discussion
5. DISCUSSION

Wistar albino rats were employed in the present study because of their small size, low cost and easy availability. The isolated rat heart preparation perfused retrogradely on Langendorff’s apparatus has been employed because changes in systemic circulation do not affect working of this preparation (Verdouw et al., 1998). Langendorff preparation and working heart preparation are hemodynamically comparable to investigate the effects of pharmacological interventions on ischemia and reperfusion–induced myocardial injury (Neely and Rovetto, 1975).

Myocardial ischemia occurs due to inadequate blood flow to the heart and, therefore, early reperfusion is necessary to restore the blood flow to the ischemic heart (Collard and Gelman, 2001). The classical ischemic preconditioning induced by four episodes of 5 min. global ischemia and 5 min. reperfusion, is reported to produce cardioprotective effect in isolated rat heart, while the delayed preconditioning was induced by administration of drugs 24 h before the isolation of heart (Fralix et al., 1993; Parikh and Singh, 1998; Parikh and Singh, 1999; Gross et al., 2008). The same protocol was, therefore, employed for classical and late preconditioning in the present study.

It has been reported that maximum release of LDH occurred immediately after reperfusion and peak release of CK-MB occurred 5 min after reperfusion (Parikh and Singh, 1998, Parikh and Singh, 1999; Sharma and Singh, 2000). Hence, samples of coronary effluents were collected at these time intervals to estimate the amount of LDH and CK-MB release which was measured spectrophotometrically using commercial kits.
The infarct size was assessed macroscopically using triphenyltetrazolium chloride (TTC) staining. (Banka et al., 1981). The NADH and dehydrogenase enzymes present in viable myocardium convert triphenyltetrazolium chloride (TTC) to red formazone pigment and stained it deep red in colour (Nachalas and Schnitka, 1963). However, infarcted cells lose dehydrogenase enzyme and cofactor NADH and thus remained unstained or dull yellow (Fishbein et al., 1981). The increase in infarct size and release of LDH and CK-MB are documented to be index of I/R-induced myocardial injury (Sharma and Singh, 2000).

Hyperglycemia was produced by single dose of streptozotocin (STZ) (50 mg/kg, i.p.) due to selective destruction of β-cells of islets of Langerhans by oxidative stress (Tanaka et al., 1995; Szkudelski, 2001). Rats with blood serum glucose level >200 mg/dl after 72 hours of administration of streptozotocin were considered to be hyperglycaemic. Experimental hyperlipidaemia was produced by feeding high fat diet (corn starch 44.74g, casein 14g, sucrose 10g, butter 20g, fiber 5g, mineral mix 3.5g, vitamin mix 1g, choline 0.25g, ter-butylhydroquinone 0.0008g, cholesterol 1g, cholic acid 0.5g) for 6 weeks (Lorkowska, et al 2006; Reeves, 1997).

Mitochondrial permeability transition pore (MPTP) is the pore of inner mitochondrial membrane, which on opening causes myocardial injury (Kroemer et al., 1998). It has been reported that MPTP opens during reperfusion injury (Griffiths and Halestrap 1995), due to oxidative stress; Ca\(^{2+}\) overload, decreased ATP levels, and increased matrix pH (Crompton et al., 1988). Opening of MPTP causes uncoupling of oxidative phosphorylation and decreases the mitochondrial ATP level (Halestrap et al., 2004). Furthermore, MPTP pore opening leads to the entry of water and solutes into the mitochondrial matrix, resulting in increased matrix volume and rupturing of outer
mitochondrial membrane (Kroemer et al., 1998). Treatment with cyclosporine-A, a MPTP inhibitor, produces cardioprotection against I/R induced injury (Griffiths and Halestrap, 1993). Opening of MPTP is also inhibited by phosphorylation and inhibition of GSK-3β (Juhaszova et al., 2004; Feng et al., 2005). Recently, it has been reported that IPC and erythropoietin produce cardioprotection by inhibiting the opening of MPTP, mediated through GSK-3β (Nishihara et al., 2007).

Thus, inhibition of the opening of MPTP is an important component in IPC mediated (Javadov et al., 2003) and cyclosporine mediated cardioprotection (Crompton et al., 1988; Griffiths and Halestrap, 1993) in rat hearts. It was reported that inhibition of the opening of MPTP by cyclosporine-A causes cardioprotection by preventing the mitochondrial overload of Ca^{2+}, indicating that IPC and pharmacological inhibition of opening of MPTP follow a common mechanism of cardioprotection. It has been reported that phosphorylation of GSK-3β, protects myocardium and IPC produces cardioprotection by phosphorylation and consequent inhibition of GSK-3β against I/R induced injury (Tong et al., 2004; Gross et al., 2004). Opening of MPTP is triggered by elevated level of calcium and of reactive oxygen species (ROS) (Gross et al., 2004; Gomez et al., 2008). It is reported that the opening of MPTP of cardiomyocytes is regulated by GSK-3β (Juhaszova et al., 2004). The threshold for ROS-induced opening of MPTP is elevated by phosphorylation of GSK-3β, and in genetically knockout GSK-3β animals, the MPTP gets easily opened in response to ROS and Ca^{2+} (Gomez et al., 2008). During IPC the threshold of MPTP opening is increased in mitochondria of cardiomyocytes (Juhaszova et al., 2004). 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 cyclophilin D (CypD), a culprit in the MPTP
opening (Nishihara et al., 2007). In isolated rat hearts, reperfusion after sustained ischemia, induces translocation of cytosolic GSK-3β to the mitochondria, where it formed a complex with ANT and VDAC.

Phosphorlylation of GSK-3β by ischemic preconditioning is dependent on protein kinase C (PKC) and phosphatidylinositol 3-phosphate kinase (PI-3K), and phospho-GSK-3β binds to ANT. Interestingly, this psho-GSK-3β-ANT interaction is associated with reduction of CypD-ANT binding and inhibition of the opening of MPTP. In addition, 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). Venkatapuram et al., (2006) showed that an inhibitor of p53, pifithrin-α, sensitized the myocardium to isoflurane-induced protection, mediated through phospho-GSK-3β. 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 et al., 2008). Suppression of ATP hydrolysis during ischemia would prevent both ATP depletion and accumulation of inorganic phosphate which promote MPTP opening.

Perfusion of the isolated rat heart with atractyloside, an opener of MPTP, during reperfusion (Jinkum et al., 2009; Park et al, 2006b), significantly attenuated the cardioprotective effect of both IPC in normal rat heart and GSK-3β inhibitor induced cardioprotection in diabetic and hyperlipidaemic rats (Yadav et al., 2010a, 2010b). This supports the hypothesis of Hausenloy et al., (2002, 2006) that the final step of
protective signaling pathways is the inhibition of opening of MPTP during reperfusion of myocardium.

In the present study, administration of single dose of STZ significantly increases the blood glucose level and feeding HL diet for 6 weeks significantly increased the plasma total cholesterol and triglycerides profile, in compression to control rats. Ischemic preconditioning reduced the I/R induced myocardial injury in term of reduction in infarct size and decrease in release of LDH and CK-MB in normal heart. In contrast, the cardioprotective effect of IPC was significantly attenuated in diabetic and hyperlipidaemic rat heart. This result is consistent with previous studies in rats (Tosaki et al., 1996; Ueda et al., 1999; Kersten et al., 2000, 2001; Ravingerova et al., 2000; Nieszner et al., 2002; del Valle et al., 2002).

The activity of Glycogen synthase kinase-3β is elevated during diabetes mellitus (Eldar-Finkelman et al., 1999; Henriksen et al., 2003). Moreover increased GSK-3β activity may cause glucose intolerance (Pearce et al., 2004) and inhibition of GSK-3β may improve 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 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). Perfusion of rat heart with GSK-3β inhibitors has been reported to produce pharmacological preconditioning (Tong et al., 2001). Furthermore GSK-3β inhibitors produced
significant protection (decreased the release of LDH and CK-MB and the myocardial infarct size) against I/R induced myocardial injury in the diabetic and hyperlipidaemic hearts. This differential effect of diabetes mellitus and hyperlipidaemia on IPC-induced and GSK-3β inhibitors-induced cardioprotection against I/R injury is a new finding in our study.

Since GSK-3β has been noted to mediate convergence of protection signaling induced inhibition of MPTP (Juhaszova et al. 2004), this selective attenuation of IPC-induced cardioprotection in the diabetic and hyperlipidaemic heart appears to be due to inhibition of the protective signaling pathways upstream of GSK-3β. Perfusion of diabetic or hyperlipidaemic rat heart with either of GSK-3β inhibitors, inhibit the opening of MPTP, by direct phosphorylating the GSK-3β and produces cardioprotection.

“Heat shock proteins” (HSP’s) are a group of proteins expressed by elevated temperature, ethanol, heavy metals, or other noxious stimuli. HSP regulate the structure of newly synthesized enzymes (Amrani et al., 1996) and are referred to as molecular chaperones. (Hartl and Martin, 1992; Csermely et al., 1998; Thirumalai and Lorimer, 2001). During cytosolic stress, the functional activity of HSF-1 get elevated which leads to the translocation of various transcription factors into the nucleus. It has been reported that the activity of various kinases such tyrosine kinases, Raf, Akt and cyclin-dependent kinases is regulated by HSP (Csermely et al., 1998; Buchner, 1999; Pratt & Toft, 2003; Zhao et al., 2005).

Under stress condition, the expression of HSP 72 is upregulated in cardiomyocytes (Lutsch et al., 1997; Manzerra et al., 1997). In human myocardium, increased hemodynamic loading through aorta banding (Delcayer et al., 1988; Knowlton et al., 105
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 et al., 1995). It has been reported that a decreasing 
cardiac content of HSP 72, may decrease the ischemic tolerance (Karmazyn et al., 
1990, Maulik et al., 1994, 1995; Yamashita et al., 1997).

Brief ischemic episodes have been noted to trigger the synthesis of HSP 72 in the 
heart, which increases the resistance against myocardial infarction after the 24 h 
(Marber et al., 1993; Zapletal et al., 2010). Heat shock has beneficial effects on 
myocardial stunning produced after a short ischemic insult. (Marban, 1991; Robinson 
et al., 1995). HSP 72 have been reported to inhibit the myocardial apoptosis by 
interaction of the SAPK/JNK signaling pathway (Gabai et al., 1997) and inhibiting 
apoptotic activity caspase 3 (Mosser et al., 1997). HSP is also noted to produce 
cardioprotection in aged animals (Snoeckx et al., 1993), against cardiac hypertrophy 
(Levy et al., 1990, Snoeckx et al., 1990) and also to improve the functional recovery 
of transplanted myocardium (Zhang et al., 1996, Mehta et al., 1997). Brief ischemic 
episodes have been reported to increases the level of HSF1 and enhance the synthesis 
of HSP 72 (Tanaka et al., 1998, Nishizawa et al., 1999; Biermann et al., 2010).

The potential of heat shock response is diminished during diabetes mellitus (Bruce et 
al., 2003), and hyperlipidemia (Cson et al., 2002). Interestingly both early and late 
phase of cardioprotective effect of ischemic preconditioning is attenuated in diabetes 
mellitus (Tosaki et al., 1996; Kersten et al., 2000; Ravingerova et al., 2000; Cson et 
al., 2002; Nieszner et al., 2002; del Valle et al., 2002) and also in hyperlipidaemia 
(Hoshida et al., 1996; Kocic et al., 1999; Udeda et al., 1999; Kyriakides et al., 2002; 
Ungi et al., 2005; Giricz et al., 2006). It has been shown that the cardioprotective
effect of preconditioning is linked to the functions of HSP 72 and the loss of the protective effect of preconditioning in these disease states is related to diminished heat stress response. The 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.

Glycogen synthase kinase-3β activity is elevated during diabetes mellitus (Eldar-Finkelman et al., 1999; Henriksen et al., 2003), while pharmacological inhibition of GSK-3β is noted to increase the expression of HSP 72 (Bijur & Jope 2000; Wang et al., 2003). To sum up, heat shock protein 72 (HSP 72) is noted to mediate late phase of cardioprotection by ischemic preconditioning (Hutter et al., 1994; Williams and Benjamin, 2000; Latchman, 2001; Snoeckx et al., 2001; Tranter et al., 2011). 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).

In present study, administration of vehicle 24 h before in diabetic and hyperlipidaemic rat, followed by 30 min of global ischemia and 120 min of reperfusion in isolated Langendorff’s heart preparation did not produce any significant effect as compared to ischemia-reperfusion control groups. However, administration of either of the GSK-3β inhibitors i.e. lithium chloride, indirubin–3 monooxime and SB216763 in diabetic and hyperlipidaemic rats, 24 h before the isolation of heart, produced significant
cardioprotection against ischemia-reperfusion-induced injury. It suggests that prior inhibition of GSK-3β is responsible for cardioprotection. The present findings are in agreement with Gross et al., (2008) that administration of SB 216763, a selective GSK-3β inhibitor, produces delayed cardioprotection in normal rat.

It has also been documented that the mechanism of preconditioning-induced-delayed cardioprotection is dependent on survival kinase pathways i.e. PI-3K/Akt (Gross et al. 2004; Gross et al., 2008) which are upstream of GSK-3β, and that phosphorylation and inhibition of the GSK-3β is responsible for cardioprotection (Gross et al. 2004).

The present results must be interpreted within the potential limitations. The half-life of SB-216763 and indirubin-3 monooxime is not clearly known. To overcome this lacuna in present study, diabetic and hyperlipidaemic rats were subjected to ischemia-reperfusion 12h and 24 h after the administration of either of GSK-3β inhibitors. The results obtained indicate that myocardium can be protected by GSK-3β, against ischemia-reperfusion- induced injury, either by immediate perfusion or after 24 h (but not 12 h) of its administration. Moreover, ischemic preconditioning mediated cardioprotection is biphasic, the early phase is of rapid onset and short duration which wane off gradually within 1 to 3 hours (Sack et al., 1993; Baxter and Ebrahim, 2002) and a late phase appears after 24 h, having a comparatively of longer duration (Bolli et al., 1998; Bolli, 2000, Baxter and Yellon, 1999). The results obtained in present study, clearly shows that the GSK-3β inhibitor-induced delayed cardioprotection closely resembles the ischemic preconditioning induced delayed cardioprotection, and that like IPC-mediated delayed cardioprotection, this delayed cardioprotective effect of GSK-3β inhibitor-induced protection appear 24 h after pre-treatment with either of GSK-3β inhibitor in diabetic and hyperlipidaemic rats.
A number of cellular processes, including transcription, metabolism, cell division, adhesion, and apoptosis are regulated by GSK-3β. Ischemic preconditioning produces delayed cardioprotection by induction of HSP 72 (Hutter et al., 1994; Williams and Benjamin, 2000; Latchman, 2001; Snoeckx et al., 2001; Biermann et al., 2010; Tranter et al., 2011) and it has been documented that HSP 72 is upregulated by pharmacological inhibition of GSK-3β (Bijur & Jope, 2000; Wang et al., 2003). In our study this observed cardioprotection was significantly attenuated by pretreatment with quercetin (4 mg/kg) an HSP 72 inhibitor in diabetic as well as hyperlipidaemic rat. This indicates that the delayed cardioprotection induced by inhibition of GSK-3β is mediating through increased expression of HSP 72. The expression of HSP 72 is diminished in diabetes mellitus (Bruce et al., 2003), hyperlipidaemia (Csont et al., 2002) and conditions leading to overexpression of GSK-3β (Chu et al., 1998).