DISCUSSION

Diabetes mellitus, hypercholesterolemia, hyperhomocysteinemia, hypertension occurs singly or may coexist in many patients. In the present study, animal models of diabetes mellitus, hyperhomocysteinemia, hypercholesterolemia, and hypertension have been used, to produce vascular endothelium dysfunction in the rat, to discern the possible role of PI3K and its downstream pathways viz PDK/AKT and PTPase, in vascular endothelium dysfunction.

Degeneration of pancreatic β cells by streptozotocin leads to insulin deficiency, followed by an increase in serum glucose level. Hyperglycemia noted in our study mimics experimental diabetes mellitus with reduced insulin secretion. Hyperglycemia associated with diabetes mellitus has been shown to impair the release of endothelial dependent relaxing factor (EDRF) and to enhance the release of endothelial dependent contracting factor (EDCF) and decrease sensitivity of vascular smooth muscle cells to nitric oxide. It also stimulates protein kinase C activity of polyol pathway, to decrease the activity and expression of eNOS (Triggle and Ding., 2010).

L-methionine administration in dose of 1.7% w/w p.o. causes methionine overloading and formation of excess homocysteine (Guilliams., 2004). Elevated homocysteine concentration, causes superoxide anion generation, hydrogen peroxide formation and oxidation of LDL, that degrades nitric oxide and reduces its concentration. Homocysteine also inhibits dimethylarginine dimethylaminohydrolase (DDAH) and consequently stimulates accumulation of asymmetric dimethyl arginine (ADMA). ADMA competes with L-arginine and decreases the formation of nitric oxide (Jakubowski., 2006).
Cholic acid stimulates absorption of cholesterol and reduces the excretion of cholesterol (Zulet et al., 1999). Therefore, high fat diet containing cholic acid was administered to rats to produce hypercholesterolemia. Hypercholesterolemia stimulates oxidation of LDL-Cholesterol, accumulation of macrophage, release of endothelins, hyperplasia of Vascular smooth muscle cells (VSMC) and generation of Reactive oxygen species (ROS), which subsequently reduces the bioavailability of nitric oxide (Bai et al., 2010).

Unilateral nephrectomy, along with high salt intake, stimulates plasma renin activity and consequently increases angiotensin II level to produce hypertension. DOCA stimulates reabsorption of water, increases blood volume, release of vasopressin, expression of endothelin 1 and alters the activity of renin angiotensin-aldosterone (RAAS) system, which consequently increases blood pressure. Therefore, uninephrectomised rats have been administered DOCA and high concentration of salt in drinking water to produce hypertension (Shah and Sing., 2006b). Hypertension stimulates the release of vasoconstrictor prostanoids, inflammatory cytokines, activates xanthine oxidase and NADPH oxidase to reduce bioavailability of nitric oxide (Sowers., 2004).

The endothelium-dependent vasodilatation was used as a parameter to assess endothelial function (Shah and Singh, 2007). Acetylcholine binds to muscarinic (M2) receptors, located on vascular endothelium to produce vasorelaxation. Cumulative dose-response curves were employed in this study, because isolated aortic ring preparation-takes a long time to relax and does not demonstrate fade phenomenon. The isolated rat aortic ring preparation used in this study offer an advantage as it avoids the risk of damage to vascular endothelium. Further, sodium nitroprusside-induced endothelium-independent vasorelaxation has been used as control in this
study, to investigate the effect of endothelium independent vascular reactivity. Increased expression of eNOS has been shown to stimulate release of nitric oxide, therefore, reverse transcription–polymerase chain reaction has been employed to assess the extent of expression of eNOS (Guido et al., 2004). Endogenous formation of nitric oxide is very unstable and gets converted to nitrate and nitrite and estimation of serum nitrite/nitrate concentration has been used as an indirect measure of nitric oxide release (Sastri et al., 2002). Thus, this parameter has been used as an index of change in nitric oxide formation due to modulation of endothelium function. Electron microscopy (EM) produces 1000X better resolution with very thin specimens (Schiller et al., 1999). Therefore, EM has been employed for examination of integrity of vascular endothelial lining of thoracic aorta.

Vascular endothelial cells regulate angiogenesis, inflammatory responses, homeostasis, as well as vascular tone and permeability. As a major regulator of local vascular homeostasis, the endothelium maintains the balance between vasodilatation and vasoconstriction (Tirziu and Simons., 2009), inhibition and promotion of the proliferation and migration of smooth muscle cells, prevention and stimulation of the adhesion and aggregation of platelets, as well as thrombogenesis and fibrinolysis (Endemann et al., 2004). Vascular endothelium releases nitric oxide which stimulates vasorelaxation and preserves the integrity of vascular endothelial lining. Dysfunction of vascular endothelium attenuates activity or expression of eNOS and consequently reduces release of nitric oxide (Dudzinski and Michel., 2007). Therefore in the present study the observed decrease in expression of mRNA of eNOS, serum concentration of nitrate/nitrite, attenuation of acetylcholine-induced endothelium dependent relaxation and disruption of integrity of vascular endothelial lining in diabetic,
hyperhomocysteinemic, hypercholesterolemic and hypertensive rats, may be due to vascular endothelium dysfunction.

Phosphatidylinositol-3-kinase (PI3K) is involved in plethora of cell signaling including endothelial cells and signaling through this enzyme is reported to be impaired in diseased states (Lindmo and Stenmark., 2006). Hallmarks of vascular metabolic function, insulin and angiotensin are also reported to act as agonists on their respective receptors and activate downstream PI3K to exert interacellular effects. Thus, insulin was employed as an agonist of tyrosine kinase receptors to activate PI3K. Furthermore a direct activator of PI3K, YS-49 was employed in the present study. Administration of both PI3K agonist insulin and YS 49 in diabetic, hypercholesterolemic, hyperhomocysteinemic and hypertensive rats significantly improved parameters employed indicating the involvement of PI3K in vascular endothelium dysfunction. The attenuation of ameliorative effect of insulin by wortmannin a specific inhibitor of PI3K further supports this contention that insulin was more effective than YS-49, may be due to pleotropic effects of insulin on vascular functions such as vasodilatation, cytoprotection and decreased glucose toxicity.

Furthermore, PDK has been reported to be one of the downstream targets of PI3K (Lemmon., 2007). Thus, UCN-01, a selective PDK inhibitor, was administered with insulin to assess its involvement. UCN-01 with insulin significantly attenuated the ameliorative effect of insulin in DM, Hch, Hhcy, HT rats indicating that insulin signaling through PDK was impaired in vascular dysfunction.

Next target enzyme to be focused was Akt, another downstream target of PI3K. Impaired membrane localization of AKT has been reported to produce vascular endothelium dysfunction in hypertension. Thus effect of API-2, a specific inhibitor of
Akt on the effect of insulin was studied. API-2 significantly attenuated the ameliorative effect of insulin, indicating that the Akt signaling may be impaired. Further, an activator of Akt, demethylasterroquinone B1 (DAQ B1) has been employed in present study. The ameliorative effect of DAQB1, very similar to insulin, confirms the involvement of AKT in DM, Hch, Hhy, HT rats.

The cellular and molecular mechanisms underlying DM, Hch, Hhy, HT rats. (Esper et al., 2006), lead to an imbalance of phosphorylation and dephosphorylation status of lipid and protein kinases that cause modulation of vascular L-arginine/nitric oxide synthetase (eNOS), to produce vascular endothelium dysfunction. The increased expression of eNOS and hence increased production of nitric oxide (Kobayashi et al., 2009), is important for cardiovascular homeostasis, vessel remodeling and angiogenesis (Dimmeler et al., 1999). Several proteins interact with eNOS and regulate its activity positively or negatively. Thus, an inhibitor of eNOS, L-NAME was employed in this study. When administered with insulin, L-NAME significantly blocked the effect of insulin, indicating that eNOS plays a significant role in insulin signaling.

Protein tyrosine phosphatases are enzymes reported to be involved in dephosphorylation insulin receptors (Shah and Singh, 2006; Topping et al., 2000) and Inhibition of PTPase has been documented to activate PI3K/PDK and AKT pathways (Papapetropoulos et al., 2004; Gao et al., 2002; Molero et al., 1998), which are known to stimulate endothelial nitric oxide synthase (eNOS) (Fleming et al., 1998). PDK is a key enzyme in PI3K/Akt signaling downstream of insulin receptors (Toker and Newton, 2000). Thus, sodium ortho vanadate (SOV), a potent and specific inhibitor of PTPase was employed to substantiate that this pathway is impaired (dephosphorylated) in experimental vascular endothelial dysfunction. Survey of
literature on in-vitro data depicts that in bovine pulmonary artery derived endothelial cell culture model, both vanadate and peroxovanadate have been shown to produce endothelial barrier dysfunction (Gilbert et al., 1998). However, in another study on bovine lung derived microvascular endothelial cells and smooth muscle co-culture, the SOV produced PI3K/AKT dependent phosphorylation of eNOS and increased the generation of nitric oxide (Papapetropolous el al., 2004). The in-vitro preparation used in our study mimics with the endothelial cells and smooth muscle co-culture and our results obtained are in agreement with the study of Papapetropolous el al.(2004). PTP-deficient mice show enhanced insulin sensitivity and are resistant to diet-induced hypercholesterolemia(Varcauteren et al., 2006). PTP inhibitors were developed as possible treatments of type II diabetes and obesity (Goldstein et al., 2000; Hooft et al., 2001). The inhibition of PTPase by specific inhibitor SOV, prevented the endothelial dysfunction in diabetes, hypercholesterolemia, hyperhomocysteinemia and hypertension, indicating thereby that increase in PTPase activity may be responsible for endothelial dysfunction. The ameliorative effect of SOV was significantly attenuated by UCN-01(PDK inhibitor) and L-NAME (inhibitor of eNOS) that shows that the effect of SOV is PDK/eNOS dependent However, UCN-01 did not block the ameliorative effect of SOV in hyperhomocysteinemia-induced vascular endothelium dysfunction. This may be due to involvement of other pathways viz oxidative stress due to autooxidation of homocysteine and MAPK.

Independent of their effect on lipid levels, statins exert pleiotropic effects, independently of its impact on LDL and cholesterol (Schulz et al., 2005) and statins have been reported to increase the generation of nitric oxide by activating eNOS through activation of PDK and Akt by prolonging the eNOS mRNA half-life (Hartmut et al., 2001). Therefore, atorvastatin was used in the present study as a
positive control, to improve vascular endothelium dysfunction Atorvastatin has been reported to ameliorate vascular dysfunction by up regulating eNOS mRNA level, increase in nitric oxide level and endothelium dependent relaxation. Atorvastatin in comparison to SOV produced somewhat greater improvement in vascular function, that may be due to its additional cholesterol lowering effect.

The activation of PI3K, PDK-1 or AKT has been reported to increase GLUT-4 mediated glucose transport (Devriese et al., 2003). Thus the observed effect of insulin, YS-49, DAQ B1, SOV and atorvastatin on serum concentration of glucose and mean arterial blood pressure may be due to activation of AKT. Further more, the observed effect of insulin and atorvastatin on lipid level and homocysteine may be due to activation of PPAR mediated biosynthesis of HDL apoA-I, decreased synthesis of cholesterol and stimulation of conversion of homocysteine to methionine and cystathione.

Limitations of the present study

1) Specific parameters such as immunohistochemistry to document the increase or decrease in the expression of phosphotidylinositol-3-kinase, protein kinase B and phosphinositide dependent kinase could not be carried out due to lack of these facility in the institution.
SUMMARY AND CONCLUSION

The present study was designed to investigate the possible involvement of pathways viz PI3K and its downstream pathway PDK/AKT in the impaired vascular endothelium dysfunction in diabetes mellitus, hyperhomocysteinemia, hypercholesterolemia and hypertension, in the rat.

On the basis of results obtained in the present study the following salient features have emerged:

1) Experimental diabetes mellitus, hyperhomocysteinemia, hypercholesterolemia and hypertension downregulated eNOS to produce vascular endothelium dysfunction.

2) Atorvastatin, a standard drug, significantly ameliorated diabetes mellitus, hyperhomocysteinemia, hypercholesterolemia and hypertension induced vascular endothelium dysfunction due to impairment of PI3K/PDK/AKT and eNOS pathways.

3) Activation of PI3K with insulin, YS 49 and AKT with demthylasteraquinone B1 significantly ameliorated vascular endothelium dysfunction. Sodium orthovanadate, a specific inhibitor of PTPase, stimulated PDK and eNOS pathways and consequently improved vascular endothelium dysfunction.

This study for the first time, reports that,

1) in addition to its metabolic effects, insulin also has a potential ameliorative effect on vascular endothelium function, the hallmark of cardiovascular system.

2) insulin, in addition to diabetes, also exerts beneficial effect in hyperhomocysteinemia, hypercholesterolemia and hypertension-induced vascular endothelium dysfunction.

3) phosphotidylinositol-3-kinase and its downstream targets viz. protein kinase B and phosphinositide dependent kinase play a significant role in insulin signaling and
signaling through this pathway is impaired in diabetes mellitus, hyperhomocysteinemia, hypercholesterolemia and hypertension induced vascular endothelium dysfunction. In addition to involvement in insulin signaling, direct activation of PI3K, PDK and AKT by their specific agonists also ameliorates vascular endothelium dysfunction.

4) protein tyrosine phosphatase may be a key enzyme dephosphorylating PDK and eNOS pathway. Thus, increasing the activity and expression of eNOS through insulin and PI3K, PDK, AKT activators may be potential therapeutic strategy to ameliorate vascular endothelial function.

CONCLUSION
Thus, it may be concluded that activation of phosphatidylinositol-3-kinase and its downstream pathways viz phosphoinositide dependent kinase/protein kinase B and endothelial nitric oxide synthetase (eNOS) improves vascular endothelium dysfunction and that therapeutic interventions designed for these pathways may provide potential therapeutic strategies to combat vascular complications.