1. INTRODUCTION

1.1. Background

Cardiovascular disease (CVD) associated public health challenges have been escalating in India and worldwide, especially in young and aging population due to genetic as well as environmental factors, including changes in lifestyle. In cardiovascular diseases, endothelial dysfunction (ED) and insulin resistance (IR) often occur as co-morbid states. Accumulating evidence from clinical, physiological, epidemiological and cellular studies demonstrate a profound relationship between ED and insulin resistance, which may lead to metabolic and cardiovascular diseases (1-7). ED is a prominent characteristic feature associated with cardiovascular disease, often associated with hypertension, atherosclerosis, type 2 diabetes (T2D), coronary artery disease and obesity, which is also characterized by insulin resistance. Currently, India has approximately 72 million adults suffering from T2D, which is estimated to nearly double by 2030.

ED reflects subtle, maladaptive alterations in endothelial cell (EC) phenotype and is manifested by reduced availability of endothelial cell derived nitric oxide (NO). Reduction in NO bioavailability supports a wide range of pathogenic events, specifically vasoconstriction, platelet activation, leukocyte adherence, thrombosis, mitogenesis, oxidation, diminished coagulation as well as vascular inflammation. Typically, insulin resistance refers to either the decreased sensitivity or responsiveness to the well-known metabolic actions of insulin, particularly the promotion of glucose uptake. On the other hand, vascular insulin resistance leads to impaired endothelial function, microvascular disease, enhanced vascular inflammation coupled with atherosclerotic lesion formation. Consequently, the impairment of the vascular insulin-signaling axis (vascular insulin resistance) may serve as one of the key triggering factor in the initiation of CVD.

The underlying cellular mechanisms in vascular insulin resistance are not completely understood. At the cell surface, the targeted binding of insulin to its cognate receptor (insulin receptor, IR) triggers two major branches of the intricate insulin signal transduction network. While phosphatidylinositol 3-kinase (PI3K) dependent signaling pathways are responsible for mediating the metabolic actions of insulin, the mitogen-activated protein kinase (MAPK) dependent insulin signaling regulates processes such as growth, mitogenesis and differentiation. The insulin signaling pathway involved in endothelial production of NO are found to be PI3K-dependent and may display striking parallels with the metabolic insulin signaling pathways in fibroblasts and adipocytes (1, 5, 6). The impairment of PI3K-dependent insulin signaling in a pathway-specific manner, contributes to a complex...
reciprocal association between insulin resistance and ED that might likely foster the clustering of metabolic and cardiovascular diseases in insulin-resistant states.

1.2. Cellular/Molecular factors inducing vascular insulin resistance

Various pathological states, including oxidative stress, dysmetabolic glucotoxicity, lipotoxicity and inflammation were found to induce impairment of the endothelial-associated insulin signalling pathway, either through the blockade of insulin receptor substrate (IRS-1), PI3K, PDK1, Akt and/or endothelial nitric oxide synthase (eNOS) activation through the inhibition of eNOS expression or through reactive oxygen species (ROS) mediated reduction of NO bioavailability (Fig. 1). Extensive research in a variety of cell types have shown that hyperglycemic conditions and chronic insulin-induced activation of Akt causes feedback inhibition of PI3K-dependent signaling. This is attributed to the sustained Akt-induced activation of the mTOR-p70S6K (S6K1)-pathway that leads to serine phosphorylation of IRS-1 and subsequently inactivation of IRS1 (8-12).

1.3. Insulin resistance and vascular inflammation

The effects of hyperglycemic conditions on endothelial cells and their role in inducing vascular inflammation are well-established. High glucose conditions lead to augmented expression of various adhesion molecules including VCAM-1, ICAM-1 and P-selectin in protein kinase C dependent signaling, resulting in the stimulation of oxidative stress and NF-kB (13, 14). Increased expression of these adhesion molecules enables leucocyte adhesion, rolling and endothelial transmigration, resulting in vascular inflammation. Similarly, the influence of inflammatory mediators on inducing insulin resistance is also understood. Multiple cytokines such as IL-1, IL-6, IL-8, IL-12, IL-18, TNF-α, TGF-β, MCP-1 and resistin have been reported to have potential involvement in the pathogenesis of insulin resistance in the context of T2D and/or
atherosclerosis (15). Studies in obese and insulin resistant models have demonstrated a clear connection between insulin sensitivity and the chronic activation of inflammatory signaling pathways. Inflammatory cytokines such as IL-1, IL-6 and TNF-α were upregulated in adipose and other metabolic tissues of obese and T2D mouse, leading to impairment of insulin signaling (16). As a consequence of hyperglycemia and hyperlipidemia, adipose tissue-resident macrophages and adipocytes produce increased levels of pro-inflammatory cytokines and release into circulation, causing vascular insulin resistance (17). Mice lacking TNF-α and TNF-α receptors were resistant to obesity-induced insulin resistance and it was observed that administration of neutralizing antibody for TNF-α in rodents improved insulin sensitivity, suggesting the influence of inflammatory mediators in inducing insulin resistance (18, 19).

1.4. Interplay between IL-6 and insulin signaling

IL-6, a proinflammatory cytokine, has previously been implicated in the pathogenesis of atherosclerosis, endothelial dysfunction and insulin resistance. A number of studies have pointed out the elevated levels of IL-6 in diabetes, obesity and CVDs (20-22). IL-6 is secreted by macrophages, B-cells, T-cells, adipose tissue, skeletal muscle, endothelium and several types of tumor cells and known to mediate a wide range of biological activities via paracrine and autocrine signalling pathways. IL-6 binds to IL-6 receptor (IL-6R), which comprises of IL-6 binding ‘a’ chain and common signal transducing ‘b’ chain-gp130 (23). On binding of IL-6 to IL-6R, two major signalling pathways are activated, namely: Janus Kinase/signal transducer and activator of transcription (JAK/STAT) as well as MAPK. JAK/STAT induces suppressors of cytokine signalling (SOCS) proteins possessing SH domains, binds to IRS1 and IR initiating their proteasome degradation, thereby, switching off insulin signalling. Interestingly, earlier studies have shown IL-6 deficient mice developed severe hepatic inflammation coupled with systemic insulin resistance (24). A recent study further demonstrated IL-6 in the secretome of Cytomegalovirus (CMV) infection during atherosclerosis induced angiogenesis via survivin and administering neutralizing antibody against IL-6 abrogated the neovascularization (25). On the other hand, IL-6 attenuated insulin mediated Akt/eNOS activity via JNK/Erk1/2 signalling axis and pharmacological inhibition of JNK/Erk reversed vasoconstrictory property of IL-6. This suggests that IL-6 impaired vasodilator effects of insulin leading to vascular insulin resistance (26). Some studies have established serum IL-6 elevation correlating to ED (27), while several other genetic analyses have hinted towards a link between polymorphism in IL-6 gene and insulin resistance in T2DM.
1.5. Epigenetic mechanisms

Epigenetic mechanisms possess a strong influence on the association between environment and gene expression. The vascular system is more prone to environmental influences owing to its development in early embryonic stage (4-5 weeks). In comparison with other organ systems, vascular system presents high flexibility in response to physiological and pathological challenges. Classical studies such as Dutch hunger study revealed environmental influence on vascular system by epigenetic reprogramming, suggesting an epigenetic interplay between genes and environment. Epigenetics is defined as a study of complex mechanisms that initiate and sustain heritable patterns of gene expression without altering the genome. There are three types of epigenetic mechanisms, including:

- DNA methylation,
- Chromatin modification, and
- Non-coding RNA-mediated pathways.

In order to regulate gene expression, these above-mentioned epigenetic pathways intervene with one another.

1.5.1. DNA methylation

DNA methylation primarily involves the covalent addition of a methyl group onto a cytosine residue located in a CpG site (i.e., where a cytosine lies adjacent to guanine in the DNA sequence). CpG islands are referred to as the regions where the CpG sites are generally clustered in high frequency near the promoter of a gene. The methylation status of CpG islands, may in turn, affect the activity and expression of the gene. DNA methylation is important in maintaining embryonic development, X-chromosome inactivation and genomic imprinting, development of plasticity and disease susceptibility.

1.5.2. Mechanism of DNA methylation

DNA methylation is reported to be catalyzed by four different types of DNA methyltransferases (DNMTs), each of which are encoded by different genes, namely: DNMT1, DNMT3a, DNMT3b and DNMT3L. De novo methylation, important in the establishment of DNA methylation during the early embryonic development phase is carried out by DNMT3a and DNMT3b. In contrast, DNMT1 serves in the maintenance and progression of DNA methylation. During the process of DNA replication, existing methylation patterns in the template strand guide the methylation of the complementary strand. DNMT3L has no catalytic domain, thus, no methyltransferase activity. However,
studies have shown that DNMT3L is responsible for DNMT3a stimulated de novo methylation.

1.5.3. Suppression of gene expression by DNA methylation

In order to repress gene expression, there are two primary mechanisms of cytosine methylation; the first mechanism involves the direct interference of methyl residue with the binding of the transcription factor to its recognition elements in the gene promoter. The lack of physical interaction between the transcription factor and the respective regulatory elements result in gene silencing. The second mechanism, however, is indirect. DNA methylation of the regions of a gene at certain density not only attracts the binding of methylated-DNA binding proteins such as MeCP2, but also recruits additional proteins such as SIN3A and histone modifying enzymes which facilitates the formation of closed chromatin configuration and silences gene expression. However, the steady-state methylation pattern is reversible and is in dynamic equilibrium with the methylase and demethylase activities. Diverse environmental factors elicit signaling pathways that subsequently affect chromatin structure as well as DNA methylation (28).

1.5.4. Epigenetic mechanisms in vasculature and insulin resistance

It is well established that epigenetic mechanisms are indispensable to the regulation of gene expression in normal and several diseased conditions. Epigenetic modifications consist of covalent modifications of the proteins that package DNA into chromatin (histone modifications include methylation, acetylation, phosphorylation, and ubiquitination), 5' methylation of the cytosine residues in CpG dinucleotides of DNA, and the gene-regulating as well as chromatin-organizing activities of noncoding RNAs. These epigenetic modifications result in varied binding of transcription activators and repressors to specific gene promoters while altering the large-scale conformation as well as function of chromatin itself, consequently modulating gene expression. The knowledge of epigenetic mechanisms in the vasculature and its implications are very scant. In vascular endothelial cells, it has been observed that transient hyperglycemia induced persistent expression of proatherogenic genes, underpinned by particular modifications in histone H3 methylation (29, 30). Genome-wide methylation analysis under hyperglycemic conditions in endothelial cells have revealed the involvement of NF-kB target genes such as heme oxygenase, MMP10, IL8 inter alia (31)
T2D is characterized by comorbid conditions such as insulin resistance and endothelial dysfunction which subsequently leads to vascular complications. Low-grade chronic inflammation is a characteristic feature of T2D. Pro-inflammatory cytokines such as IL-6 have been demonstrated to disrupt insulin signalling and leads to vascular insulin resistance. Bidirectional activation between epigenetic modifications and endogenous (inflammation, hyperglycemia)/exogenous factors (lifestyle) govern onset and progression of T2D and associated vascular complications. However, the interplay between inflammation and epigenetics in the context of vascular diseases is not well understood. Hence, the present day study is focused on understanding the cross talk between IL-6 and insulin signalling pathways and underlying epigenetic changes in human vascular endothelial cells.