2.1. Diabetes Mellitus

Diabetes mellitus (DM) is regarded as a metabolic disorder characterized by hyperglycemia linked with disturbances in carbohydrate, fat and protein metabolism resulting from a deficiency of insulin release, an inefficient function of insulin or both (Baquer et al., 1998). In the year 1965, World Health Organization (WHO) was first put forward the diagnostic criteria and the classification of diabetes (1965), later by the National Diabetes Data Group in 1979 (1979), and subsequently followed by WHO simplified recommendations in 1980 (1980) and 1985 (1985). In 1997, American Diabetes Association (ADA) had been published the latest recommendations (1997). World Health Organization was agreed on the recommendations and criteria in 1999 (Alberti & Zimmet, 1998).

For the diagnosis of diabetes, the ADA recommendations are as follows:

- The fasting plasma glucose should be routinely used for screening of diabetes as well as epidemiological studies
- Symptoms of diabetes such as polydipsia, polyurea, sudden weight loss etc.
- The threshold value for fasting glucose was reduced from 7.8 mmol/L (140 mg/dl) to 7.0 mmol/L (126 mg/dl), with no caloric intake for at least 8 h.
- 2-h plasma glucose criterion after ingestion of 75 g anhydrous glucose in water should be 11.1 mmol/L (200 mg/dl).

DM is categorized into two major types: type 1 DM (T1DM) and type 2 DM (T2DM). Based on the aetiology, T1DM and T2DM were used to describe insulin dependent DM and non-insulin dependent DM, respectively.

2.2. Epidemiology of diabetes

According to the International Diabetes Federation, there were an estimated 382 million people affected with diabetes globally in 2013, expected to reach 592 million by 2035 (**Table 2.1**). The burden of diabetes is rising rapidly in every country, fuelled by the worldwide increase in the incidence of obesity and unhealthy dietary habits.

Table 2.1: Top ten countries for number of adult population (aged 20–79 years) with diabetes in 2013 and 2035.

Year	**2013**		**2035**	
S.No.	**Country**	**No. of adults with diabetes (millions)**	**Country**	**No. of adults with diabetes (millions)**
1	China	98.4	China	142.7
2	India	65.1	India	109.0
3	USA	24.4	USA	29.7
4	Brazil	11.9	Brazil	19.2
5	Russian Federation	10.9	Mexico	15.7
6	Mexico	8.7	Indonesia	14.1
7	Indonesia	8.5	Egypt	13.1
8	Germany	7.6	Pakistan	12.8
9	Egypt	7.5	Turkey	11.8
10	Japan	7.2	Russian Federation	11.2

Adapted from International Diabetes Federation, Diabetes Atlas Sixth Edition (www.idf.org/diabetesatlas), 2013, Brussels.

T1DM and T2DM are widely accepted aetiological forms of diabetes; however, type 2 diabetes is responsible for the majority (>85%) of total prevalence of diabetes. The exact time of onset of T2DM is hard to determine, and up to one-half of diabetic patients remain undiagnosed because of its slow onset nature and the absence of acute metabolic disturbance seen in T1DM. Furthermore, different factors like genetic predisposition, ethnicity, lifestyle changes, and globalization contribute to the rapid prevalence of diabetes in India. Indian Council of Medical Research (ICMR) conducted

an extensive community study and reported that a less population is affected in states of Northern India (Jharkhand 0.96 million, Chandigarh 0.12 million) as compared to Tamil Nadu (4.8 million) and Maharashtra (9.2 million) (Anjana et al., 2011). Thus, there is a noticeable geographical variation in the prevalence of diabetes, but with a different pattern.

Two population-based studies like CURES (Chennai Urban Rural Epidemiology Study) (Mohan et al., 2006a) and CUPS (Chennai Urban Population Study) (Mohan et al., 2006b) provide clear cut idea about the prevalence of diabetic complications in India. In the CURES study, the overall prevalence of diabetic retinopathy, overt nephropathy, microalbuminuria, proteinuria and peripheral neuropathy were 17.6% (Rema et al., 2005), 2.2% (Unnikrishnan et al., 2007), 26.9% (Unnikrishnan et al., 2007), 19.7% (Ramachandran et al., 1999) and 26.1% (Pradeepa et al., 2008) respectively. The CUPS study reported that coronary artery disease (CAD) was prevalent in 9.1% of patients with normal glucose tolerance, 14.9% of patients with impaired glucose tolerance, and 21.4% of patients with diabetes (Mohan et al., 2001). Additionally, diabetic patients also showed an increased intimal medial thickness of carotid artery (subclinical atherosclerosis), compared to non-diabetic patients (Mohan et al., 2000). Thus, the increased morbidity and mortality in diabetic patients could be attributed to an increased burden of diabetic macro- and microvascular complications. Therefore, the morbidity, mortality, increased financial burden, inadequate health care, and reduced life expectancy make diabetes a significant public health condition.

2.3. Diabetic Cardiomyopathy

Although T1DM and T2DM differ in aetiology and metabolic profiles, the two types share many features of cardiomyopathy. Rubler et al. (1972) was observed no known cause for heart failure in diabetic glomerulosclerosis patients and first introduced the concept of "Diabetic cardiomyopathy" (Rubler et al., 1972), which is characterized by alterations in the morphology and functions of the diabetic heart without hypertension or coronary heart disease (Aneja et al., 2008). Perivascular and interstitial fibrosis is a distinctive feature of diabetic cardiomyopathy (Rubler et al., 1972; van Hoeven & Factor, 1990), and the heart weight correlates with the extent of fibrosis (van Hoeven & Factor, 1990). Apart from the increased deposition of collagen, diabetes enhances the cross-linking of collagen fibres and leads to diminished ventricular compliance (Goldin et al., 2006). Hearts of diabetic patients demonstrated increased interstitial fibrosis and cardiomyocyte size compared with hearts of non-diabetic patients when biopsied at the time of coronary bypass surgery (Fischer et al., 1984). T1DM and T2DM animal models demonstrated an enhanced cross-sectional area of cardiomyocytes with or without interstitial fibrosis (Li et al., 2010; Sakata et al., 2007). However, a marked decrease in cardiomyocytes cross-sectional area was also observed in Akita (Ins2WT/C96Y) mouse, a T1DM model (Basu et al., 2009). Thus, overall, myocardial hypertrophy seems to be a recurrently observed phenotype but not an essential feature of diabetic cardiomyopathy and can be speculated that diabetes-induced modification of microcirculation and chronic metabolic derangements resulting into varying levels of atrophy, hypertrophy and cardiomyocyte loss depending on the presence of diabetic comorbidities such as hypertension. Although it has not been involved in the classification of diabetic cardiomyopathy, enhanced sensitivity of diabetic hearts to ischemia-reperfusion injury may be an essential aspect of diabetic cardiomyopathy.

Clinical studies indicated that diabetic patients had greater 30-80% cardiac infarct size following coronary reperfusion intervention than non-diabetic patients (Alegria et al., 2007; Marso et al., 2007), and the difference was more evident even after similar percutaneous coronary intervention (Marso et al., 2007).

2.3.1. Morphology and functional alterations of heart in diabetic cardiomyopathy

Numerous experiments have demonstrated that T2DM is consistently linked with concentric left ventricle (LV) remodeling or hypertrophy (i.e. can be calculated by the ratio of LV mass [LVM] to LV end-diastolic volume) in females but not in males (Bella et al., 2001; Rutter et al., 2003), however, the rise of LVM and the ratio of LVM to LV end-diastolic volume is significantly associated with insulin resistance and hyperglycemia regardless of gender and age (Heckbert et al., 2006). Dysregulated diastolic function with average ejection fraction of LV is a common echocardiographic finding of T1DM and T2DM patients. Boyer et al. (2004) reported that 46%-63% of asymptomatic T2DM patients exhibited LV diastolic dysfunction (Boyer et al., 2004). Although LV diastolic dysfunction predominant in diabetic cardiomyopathy, LV systolic dysfunction is also a part of it as diabetes also impairs LV systolic function. Diabetic patients had lowered values of LV fractional shortening (LVFS) than those control subjects with normal glucose tolerance (Bella et al., 2001; Heckbert et al., 2006). Marked dysfunction of LV was also demonstrated by T1DM patients (Gul et al., 2009). Taken together, subendocardial dysfunction reflected by impaired longitudinal LV shortening is considered as one of the initial symptoms of diabetic cardiomyopathy.

2.3.2. Dysfunction of excitation-contraction coupling of diabetic myocardium

Contractile dysfunction of the heart was observed in different animal models of T1DM (e.g., alloxan-treated and streptozotocin-treated animals) and T2DM (e.g., Otsuka-Long-Evans-Fatty rat, Goto-Kakizaki rat, ob/ob mouse). Diabetes markedly alters the $Ca2^{+}$ transient, action potential and $Ca2^{+}$ sensitivity of myocardial contractile elements (Lacombe et al., 2007). Before the development of systolic ventricular dysfunction, diabetic cardiomyocytes exhibit some notable changes like the slower decay of $Ca2^{+}$ transient and prolongation of action potential duration. It is demonstrated that peak amplitude of $Ca2^{+}$ transient was diminished in diabetic animal models (Lacombe et al., 2007), in addition to decreased L-type $Ca2^{+}$ channel expression. Delayed decay in $Ca2^{+}$ transient in the diabetic heart can be due to decreased affinity of troponin C for $Ca2^{+}$ (Ishikawa et al., 1999), reduced sarcoplasmic reticulum $Ca2^{+}$ ATPase 2a (SERCA2a) protein level (Belke & Dillmann, 2004), enhanced phosphorylation of phospholamban (Pereira et al., 2006), nonenzymatic glycosylation of SERCA2a (Bidasee et al., 2004) and restored the function of Na^{+}-$Ca2^{+}$ exchanger (Zhang et al., 2008).

Reduced expression of the voltage-gated K^{+} channel (Kv4.2) also leads to decreased outward K^{+} current (Ito) in diabetic hearts. The proposed mechanisms responsible for reduced Ito current are stimulation of peroxisome proliferator-activated receptor-α (PPAR-α), inhibition of pyruvate dehydrogenase (PDH) (Huang et al., 2003). PPAR-α activation stimulates pyruvate dehydrogenase kinase 4 (PDK4) which inhibits PDH activity (Sugden & Holness, 2006), and chronic stimulation of cardiac-specific PPAR-α has been demonstrated to decrease Ito current density and the protein expression of Ito channel by downregulating both α-subunit and β-subunit (Marionneau et al., 2008).

Hence, in diabetes, stimulation of PPAR-α by enhanced fatty acid uptake leads to downregulation of Kv4.2 channels by inhibiting PDH activity.

2.3.3. Metabolic derangements in diabetic myocardium

ATP turnover is up to 35 kg/day, which is several times larger than its synthesis, and energy utilization from substrates is not considerable (up to 25%) in the heart (Knaapen et al., 2007). Therefore, even a little decrement in the ATP production efficiency could markedly lead to dysfunction of myocardial contraction and relaxation, which is a highly energy-dependent process. Diabetes diminishes the energy production efficiency of cardiomyocytes through inhibition of glucose oxidation and enhancing fatty acid uptake. Diabetes-induced PPAR-α activation further increases fatty acid oxidation by upregulating several fatty acids metabolizing enzymes (Hafstad et al., 2009). Furthermore, activation of PPAR-α leads to upregulation of PDK4 transcription, thereby attenuates glucose oxidation (Buchanan et al., 2005). Besides, several steps lead to decreased glucose oxidation in the diabetic myocardium are: reduced glucose uptake by reduced expression of GLUT1/GLUT4 receptors (Desrois et al., 2004), blunted PI3K-Akt signalling pathway, impaired tyrosine phosphorylation of insulin receptor, suppression of enzymatic activities of PDH, phosphofructokinase and glucokinase by PDK4, citrate and long-chain acyl-CoA (Heather & Clarke, 2011).

The impaired mitochondrial function is responsible for the decreased efficiency of cardiomyocytes energy production and increased production of reactive free radicals in diabetic heart (Bugger & Abel, 2010). Higher levels of oxygen utilization due to enhanced fatty acid oxidation and increased function of mitochondrial uncoupling proteins (UCP3) seems to trigger the increased synthesis of ROS and reduced the mitochondrial efficiency of ATP production (Echtay et al., 2002). It has also been

reported that extra-mitochondrial Nox-derived ROS levels are also elevated in obese Zucker fatty rat model of T2DM due to upregulation of NADPH activity by an increased catalytic function of glucose-6-phosphate dehydrogenase (Serpillon et al., 2009).

2.3.4. Remodeling of extracellular matrix in diabetic hearts

Glycated proteins undergo cross-linking to each other and form complex compounds like advanced glycation end products (AGEs), and hyperglycemia triggers the plasma levels of AGEs (Goldin et al., 2006). Deposition of AGE in collagen facilitates cross-linking of collagen and decreases collagen hydrolytic turnover (Verzijl et al., 2000), and resulting in enhanced stiffness of arteries and diabetic heart. Furthermore, plasma levels of AGE positively correlate with arterial stiffness, isovolumetric relaxation time and carotid intimal thickness (Yoshida et al., 2005). In addition, fibrosis in the cardiac perivascular space and interstitium is more evident during late stages of diabetic animal models and indeed related to increased angiotensin receptor (AT1) activity (Hotta et al., 2010).

2.3.5. Abnormalities in the microvasculature

"Microangiopathy" (disease of small blood vessels) has been reported in diabetic hearts (Factor et al., 1980) and morphology of microvessels in myocardium demonstrated spasm, microaneurysms and spiral deformation (Factor et al., 1980). In addition to morphology, Yoon et al. (2005) showed that reduced capillary density and increased interstitial fibrosis and apoptotic cell death of endothelial cells are closely associated with downregulated expression of vascular endothelial cell growth factor (VEGF) in diabetic heart (Yoon et al., 2005). Diabetes promotes a reduction in coronary blood flow

reserve (CFR) (Mizuno et al., 2010; Yu et al., 2002), and CFR has been negatively associated with an index of LV relaxation (Mizuno et al., 2010). In obese type 2 diabetic rats, CFR was decreased and inversely related to the extent of perivascular fibrosis and the wall-to-lumen ratio of arterioles (Yu et al., 2002). Stimulation of endothelial RAGE by AGE suppresses the NO synthesis and increases the expression of cell adhesion molecules, and alagebrium (an inhibitor of AGE cross-link formation) markedly enhanced flow-mediated dilatation in hypertensive patients (Zieman et al., 2007). Thus, AGE-mediated coronary media stiffening, decreased NO synthesis, perivascular fibrosis and reduced angiogenesis are possibly leading to the reduced CFR in diabetic myocardium.

2.3.6. Diabetes-induced changes in myocardial tolerance against ischemia-reperfusion (IR) injury

Although clinical trials reported that diabetic patients treated with reperfusion therapy demonstrated enlargement of infarct size (Alegria et al., 2007; Marso et al., 2007), preclinical experiments have also shown the differential impact on infarct size due to diabetes induction (Ma et al., 2006; Xu et al., 2004). The discrepancy in results may due to multiple differences in the protocols employed; different plasma insulin levels (i.e. T1DM vs T2DM) and duration of diabetes. In a study, one-week diabetic rat hearts limited the cardiac infarct size after 30-min ischemia, but the infarct size limitation was not evident in 8 weeks diabetic rat hearts (Ravingerova et al., 2003). Two other experiments also supported that diabetic hearts demonstrated increased resistance against IR injury during initial stages of diabetes; however, resistance was disappeared during later stages of diabetes (Ma et al., 2006; Xu et al., 2004). In contrast to this, enlargement of infarct size in 8 days diabetic rat hearts was also reported (Marfella et al., 2002), suggesting the role of other factors influencing infarct size, irrespective of the

duration of diabetes. As for insulin role in infarct size change, diabetic models with hyperinsulinemia and obesity (Bouhidel et al., 2008; Hotta et al., 2010; Miki et al., 2009) demonstrated larger infarct size than their counterparts.

2.4. Mechanisms Responsible for Diabetic Cardiomyopathy

2.4.1. Oxidative, nitrosative and nitrative stress

The two primary mechanisms responsible for damage to the myocardium and leading to detrimental diabetic cardiovascular complications are oxidative stress and nitrosative stress (Giacco & Brownlee, 2010; Jay et al., 2006). Diabetic milieu like persistent hyperglycemia, accumulation of AGEs, glucose autoxidation, increased receptors for AGE (RAGE), enhanced level of leptin and free fatty acids, plays a vital role in the production of reactive oxygen and nitrogen species (ROS/RNS) in diabetic myocardium (Giacco & Brownlee, 2010; Jay et al., 2006) (**Figure 2.1**). Persistent hyperglycemia in both T1DM and T2DM (Boudina et al., 2005; Wold & Ren, 2004) is connected with the enhanced production of ROS and RNS in mitochondria, a significant source of free radical generation. Superoxide radicals generate as an inevitable by-product through mitochondrial electron transport chain process at complex I and complex III. ROS causes damage to cardiac cells through various mechanisms (interference with nitric oxide (NO), oxidation and enhancing detrimental signalling pathways).

Several cellular and subcellular enzymes such as xanthine oxidase/oxidoreductase (Rajesh et al., 2009), nicotinamide adenine dinucleotide phosphate oxidases (NADPH oxidases) (Rajesh et al., 2012), uncoupled NO synthase (NOS) (Pacher et al., 2007), microsomal enzymes and the mitochondrial respiratory chain (Giacco & Brownlee, 2010), that may account for increased ROS generation in the diabetic heart. Among

these, elevated mitochondrial ROS production was considered as the primary source of hyperglycemia-induced oxidative stress in endothelial cells (Camici et al., 2007) and cardiomyocytes (Giacco & Brownlee, 2010). There is also support for enhanced ROS production from non-mitochondrial sources. NADPH oxidases are specialized enzymes responsible for the generation of vast amounts of superoxide and hydrogen peroxide radicals under different pathological diseases (Maalouf et al., 2012). In the heart and aorta of Zucker diabetic fatty rats, NADPH activity is further amplified by the enhanced production of NADPH by glucose-6-phosphate dehydrogenase (Serpillon et al., 2009). Vascular NADPH oxidase (a downstream molecule of angiotensin II) also plays a pivotal role in the development of diabetic cardiovascular complications (Fiordaliso et al., 2000; Westermann et al., 2007a). It was evident that renin-angiotensin system is activated in streptozotocin-induced type 1 diabetes and AT1 receptors are overexpressed in diabetic hearts or cardiomyocytes exposed to high glucose (Fiordaliso et al., 2000; Westermann et al., 2007a). Simultaneously, enhanced stimulation of XO, another ROS generating enzyme, has also been demonstrated in diabetic heart (Rajesh et al., 2009). Oxidative damage or deficiency of anti-oxidant system could activate a variety of responses related with ventricular remodellings, such as modulation of signalling pathways associated with apoptosis (Takano et al., 2003) and myocardial hypertrophy (Cesselli et al., 2001), stimulation of matrix metalloproteinase to alter the structure of extracellular matrix (King et al., 2003). Therefore, enhanced ROS leads to myocardial dysfunction by oxidative modification of proteins and DNA, and by inducing cell death.

Oxidation of proteins can be caused directly by ROS or by lipid peroxidation (e.g., 4-hydroxynonenal or malondialdehyde) and results in dissociation of catalytic subunits of enzymes, aggregation or fragmentation. In fact, oxidative posttranslational

modifications are essential for the proteins (LC3-II and Atg4) involved in autophagosome formation and maturation (Scherz-Shouval et al., 2007). Thus the functional properties of these proteins are also intensely affected by redox imbalance (Scherz-Shouval et al., 2007) and significantly contribute to myocardial dysfunction. In diabetic hearts, oxidative protein modifications involved in antioxidant defence, contractility, protein folding, metabolism (mostly glucose and fatty acid), excitation-contraction coupling, and calcium handling, which play a crucial role in the development of diabetic myocardial dysfunction.

Substantial evidence indicates that decreased availability of NO and its signalling in diabetic tissues are related to the modulation of endothelial NOS activity (Pacher et al., 2007). Uncoupling of NOS (i.e. dimerization to monomerization) resulted in decreased NO bioavailability (since monomeric form favours the formation of superoxide anion instead of NO) and enhanced oxidative stress, which has been implicated in the pathophysiology of different cardiovascular ailments. In addition to uncoupling of myocardial NOS isoforms, the expression of eNOS and iNOS has been reported to be enhanced (especially iNOS form) in the diabetic myocardium (Rajesh et al., 2012). The augmented expression of NOS in the diabetic heart is linked with enhanced levels of 3-nitrotyrosine formation (a marker of peroxynitrite production and nitrative stress) and lipid peroxidation, which might be associated with the monomer state or uncoupling of the enzyme, and trigger the formation of peroxynitrite by releasing superoxide radicals instead of NO. Superoxide anion combines with NO indeed forms peroxynitrite, a robust cytotoxic oxidant, damages various biological molecules via numerous mechanism (Pacher et al., 2007). Intriguingly, peroxynitrite itself also catalyzes uncoupling of NOS by targeting or disrupting caveolae-NOS association (Cassuto et al., 2014). Peroxynitrite levels not only induces necrotic or apoptotic cell death in

endothelial cells and cardiomyocytes via stimulation of poly ADP ribose polymerase 1 (PARP-1) or mitogen-activated protein kinase (MAPK)-dependent pathways, but also promotes nitration and consequent suppression of crucial proteins responsible for energy homeostasis, intracellular calcium cycling, myocardial contractility and antioxidant defense (Pacher et al., 2007). Peroxynitrite may also impair NO-dependent guanylate cyclase signalling pathway and render NO to unable to stimulate its primary protective pathway (Evgenov et al., 2006) and also activate matrix metalloproteinases (MMPs) in the failing hearts to support pathological remodelling (Pacher & Szabo, 2006; Rajesh et al., 2010). The persistently enhanced cardiac oxidative and nitrative stress in diabetic hearts leads to depletion of endogenous antioxidants (e.g. glutathione and metallothione) (Rajesh et al., 2010), dysregulation of crucial antioxidant defence system (Pacher et al., 2007) (e.g. suppression of catalase and superoxide dismutase), and dysfunction of redox-dependent transcription factors (e.g. Nrf2) (Li et al., 2012).

2.4.2. Inflammation

The substantial evidence demonstrated that a chronic cardiac inflammation in both type 1 and type 2 diabetes is linked to the development of diabetic cardiomyopathy (Boudina & Abel, 2007; Prabhu & Frangogiannis, 2016). Tschope et al. (2005) showed diabetic cardiomyopathy phenotype using streptozotocin-induced type 1 diabetic rat model, which was characterized by significant increases in interleukin 1 beta (IL-1β), tumor necrosis factor alpha (TNF-α), expressions of vascular cell adhesion molecule 1 (VCAM-1) and myocardial intracellular adhesion molecule 1 (ICAM-1) and beta2-leukocyte-integrins (CD11a+, CD11b+, CD18+) which were directly correlated with elevated oxidative stress and left ventricular dysfunction (Tschope et al., 2005). Intriguingly, transgenic stimulation of kallikrein-kinin system repressed cardiac

inflammation, oxidative stress and endothelial dysfunction in diabetic hearts (Tschope et al., 2005). Moreover, it has been reported that genetic deletion of kinin receptor B (Westermann et al., 2009) or neutralization of TNF-α (Westermann et al., 2007b) attenuated the development of diabetic cardiomyopathy associated with a decline in cardiac inflammation and fibrosis in rat or mouse models of type 1 diabetes. Similarly, IL-6 knockout mice showed decreased fibrosis and myocardial inflammation, and improved heart function in response to streptozotocin-induced type 1 diabetes, through the downregulation of downregulation of TGF-β and upregulation of microRNA-29 (Zhang et al., 2016c). This favourable effect was connected to AMP-activated protein kinase (AMPK) stimulation and the abolition of suppressor of cytokine signalling 3 (SOCS3)-mediated inhibition of insulin receptor substrate (IRS)-1 (Ko et al., 2009).

Recently, several studies have been reported that stimulation of nuclear factor kappa B (NF-κB) in diabetic myocardium (Rajesh et al., 2010) or human cardiomyocytes exposed to high glucose concentrations triggered the excessive production of ROS/RNS (Rajesh et al., 2010). NF-κB stimulates the expression of pro-inflammatory cytokines, such as IL-6, pro-IL-18, pro-IL1-β and TNF-α in the heart. Cytokines can decrease ventricular contractile function and cardiac cell viability through several mechanisms, however, majorly through the formation of peroxynitrite (Levrand et al., 2006). Other proposed mechanisms like modulation of sarcoplasmic reticulum calcium ATPase expression (Nian et al., 2004) and regulation of extracellular matrix composition and dynamics in heart (Li et al., 2000) may also be responsible for detrimental effects of cytokines. Of note, NF-κB can also activate the nucleotide-binding oligomerization domain-like receptor protein 3 (NLRP3)-inflammasome (Fuentes-Antras et al., 2014).

Interestingly, the role of NLRP3-dependent inflammasome in the development of insulin resistance, diabetes and diabetic cardiomyopathy has also been investigated. Inhibition of NLRP3 inflammasome *in-vivo* reduced type 2 diabetes-induced fibrosis, myocardial inflammation and improved ventricular function (Luo et al., 2014). NLRP3 was found to be stimulated in the liver and adipose tissue in response to obesity. Pharmacological inhibition or genetic ablation of NLRP3-inflammasome was seen to diminish obesity-induced insulin resistance and diabetes (Vandanmagsar et al., 2011; Yan et al., 2013). Knockdown of NLRP3 decreases mature IL-1β expression and improves cardiac function in diabetic rat models with a combination of high-fat diet and streptozotocin injection (Luo et al., 2014).

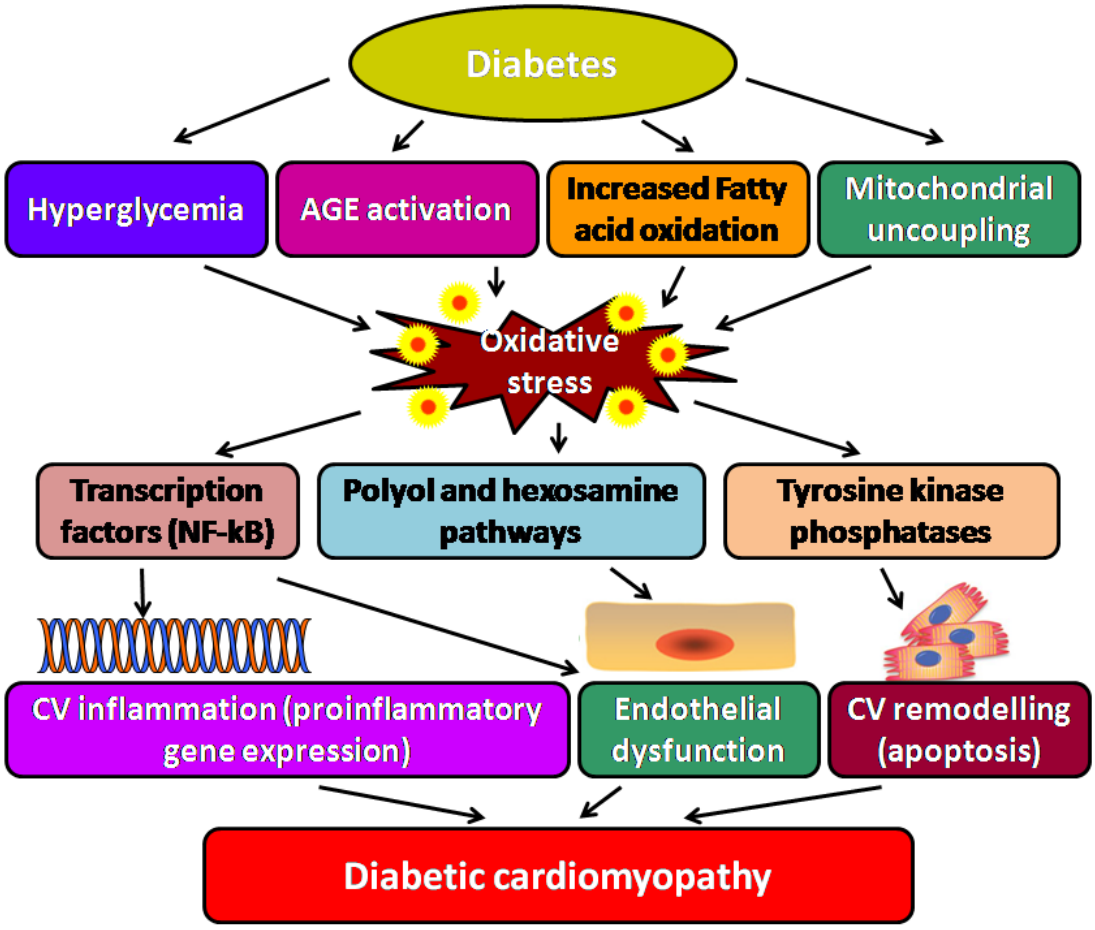


Figure 2.1: Pathological mechanisms responsible for diabetic cardiomyopathy.

In diabetes, hyperglycemia, AGE activation, increased fatty acid oxidation, mitochondrial uncoupling enhances oxidative stress, which in turn renders the myocardium more vulnerable to damage by promoting inflammation, endothelial dysfunction and remodeling through upregulation of NF-κB, polyol-hexosamine pathways and tyrosine kinase phosphatases respectively.

2.4.3. Defective intracellular protective mechanisms in diabetes

Diabetes causes the deregulation of survival signalling pathways for myocardial protection (**Figure 2.2**). The substantial evidence demonstrated that phenomena like ischemic preconditioning or postconditioning lost their cardioprotective nature in diabetic condition (Bouhidel et al., 2008; Katakam et al., 2007). Similarly, diabetic clinical subjects demonstrated deficient cytoprotective mechanisms in hearts. For example, T2DM rats demonstrated suppressed JAK2/PI3K/Akt signalling via enhanced calcineurin activity and dysfunction of ERK/GSK3β signalling through increased endoplasmic reticulum stress (Hotta et al., 2010; Miki et al., 2009). Moreover, increased protein levels of GSK3β in mitochondria leads to enhanced mitochondrial permeability transition in response to $Ca2^{+}$ overload (Miki et al., 2009). There is a limited data on whether glycemic control normalizes the deficient cardioprotective signalling pathways in diabetic hearts. Dextrose-induced acute hyperglycemia prevented the cardiac infarct size limitation by anaesthetic agents, ischemic preconditioning and mitochondrial K+ATP channel opener (Amour et al., 2010; Kersten et al., 1998), suggesting a promising role of hyperglycemia in diminishing survival pathways.

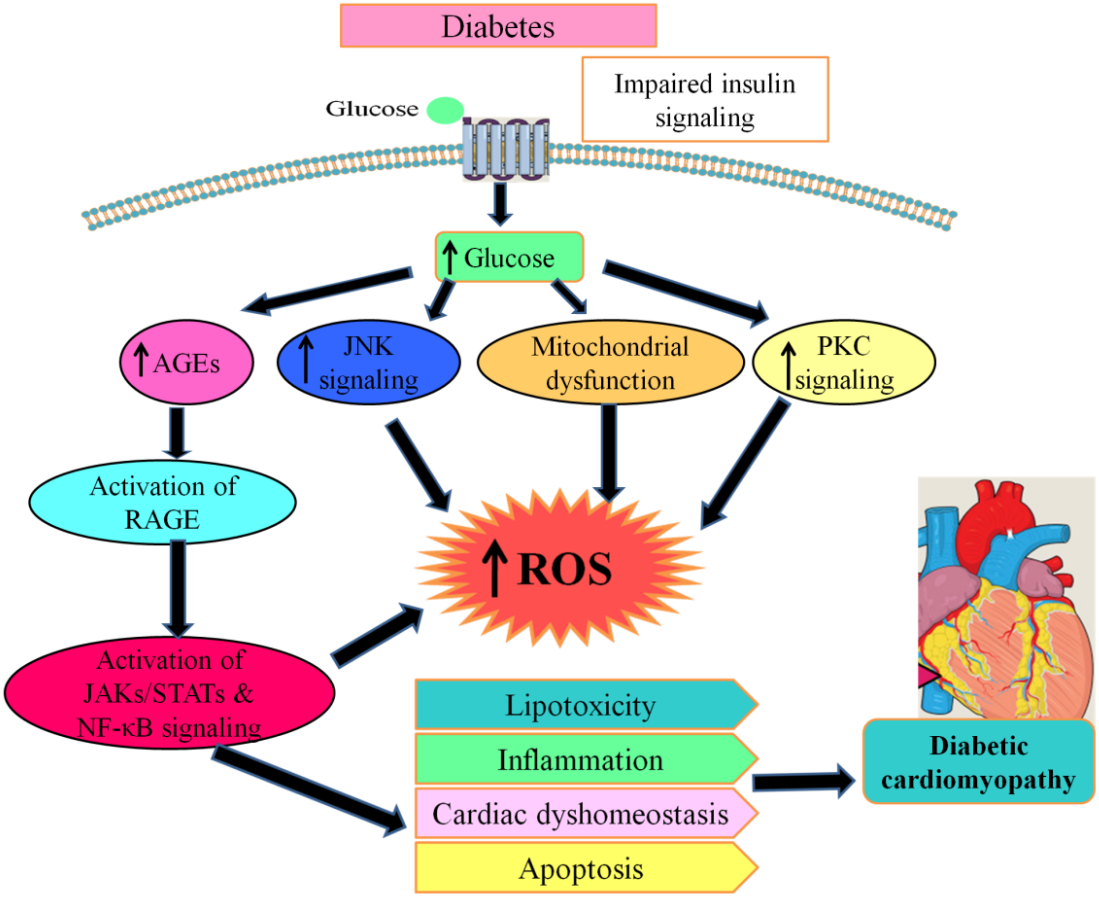


Figure 2.2: Defective signalling pathways in diabetic cardiomyopathy.

Hyperglycemia and impaired insulin signalling alters mitochondrial function and several cell signalling pathways such as JAK/STAT, NF-κB, JNK, and PKC. ROS are responsible for lipotoxicity, inflammation, cardiac dyshomeostasis, and apoptosis, thereby leading to diabetic cardiomyopathy. ↑ indicates elevated levels or expression.

2.5. Principal Targets for the Prevention of Diabetic Cardiomyopathy

The biological pathways regulating energy homeostasis, oxidative stress and inflammation have been targeted for pharmacological treatment to combat diabetes, and its associated cardiac complications involve AMP-activated protein kinase (AMPK), nuclear factor-erythroid (NF-E) 2-related factor 2 (Nrf2), hemeoxygenase (HO-1).

2.5.1. AMP-activated protein kinase (AMPK)

AMPK exists as a heterotrimeric enzyme, enclosing catalytic α and regulatory β, γ subunits (Davies et al., 1994). AMPK is extensively distributed, and two α isoforms (α1, α2), two β isoforms (β1, β2) and three γ isoforms (γ1, γ2, γ3) of AMPK have been recognized till date (Hardie & Hawley, 2001). Mostly, α2 isoform is essential for cardiac AMPK function under basal and stress situations (Russell et al., 2004b). The α2 isoform is most predominant (contributing 70-80% of total AMPK activity) within the heart (Li et al., 2006). The β subunit contains glycogen-binding domain and acts as a bridge between α and γ subunits (Polekhina et al., 2003). The γ subunit includes four tandem C-terminal cystathionine beta-synthetase domains which bind AMP and critical for AMPK regulation (Scott et al., 2004). Although AMP is involved in allosteric activation of AMPK, it has recently been found that ADP can regulate AMPK activity by phosphorylation and dephosphorylation (Oakhill et al., 2011).

Activation of AMPK by upstream kinases largely depends on phosphorylation of the Thr172 residue of catalytic α subunit (Kemp et al., 2007). Several upstream kinases such as LKB1 (Sakamoto et al., 2006), calcium/calmodulin-dependent kinase kinase β (CaMKKβ) (Hawley et al., 2005), transforming growth factor-β-activated protein kinase-1 (TAK1) (Xie et al., 2006), phosphorylates and regulate AMPK function. Whereas Protein phosphatases (PP) such as PP2A and PP2C involved in dephosphorylation of AMPK (Sanders M et al., 2007).

AMPK has been shown to exhibit both pro-apoptotic and anti-apoptotic actions in cardiomyocytes; however, overwhelming cardiac studies suggested that AMPK stimulation is antiapoptotic. Capano & Crompton (2006) demonstrated proapoptotic effects of AMPK are mediated by mitochondrial translocation of BAX (Capano &

Crompton, 2006). In contrast to this, Kewalramani et al. (2009) have shown that stimulation of AMPK strikingly prevented TNF-α-induced cardiomyocytes apoptosis and is mediated by promoting BAD phosphorylation (proapoptotic protein) and eventually inhibiting mitochondrial apoptotic signalling events like cytochrome c release and subsequent activation of caspase 3 by restricting its association with BCL-XL (antiapoptotic protein) (Kewalramani et al., 2009). Similarly, AMPK stimulation is indispensable in offering protection against oxidative stress-induced apoptosis in H9C2 rat cardiomyocytes (Sasaki et al., 2009) and palmitate-induced apoptosis in neonatal cardiomyocytes (Hickson-Bick et al., 2000). Russell et al. (2004) showed that AMPK activation is beneficial in decreasing apoptosis in the ischemic heart of transgenic mice expressing the kinase-dead mutant of AMPKα2 mostly by improving metabolic effects like glucose uptake and glycolytic flux (Russell et al., 2004b). Besides, Shibata et al. (2005) demonstrated the antiapoptotic function of adiponectin against myocardial ischemia/reperfusion is mediated by AMPK activation (Shibata et al., 2005). Thus, AMPK plays a crucial role in limiting cardiac apoptosis associated with IR (**Figure 2.3**).

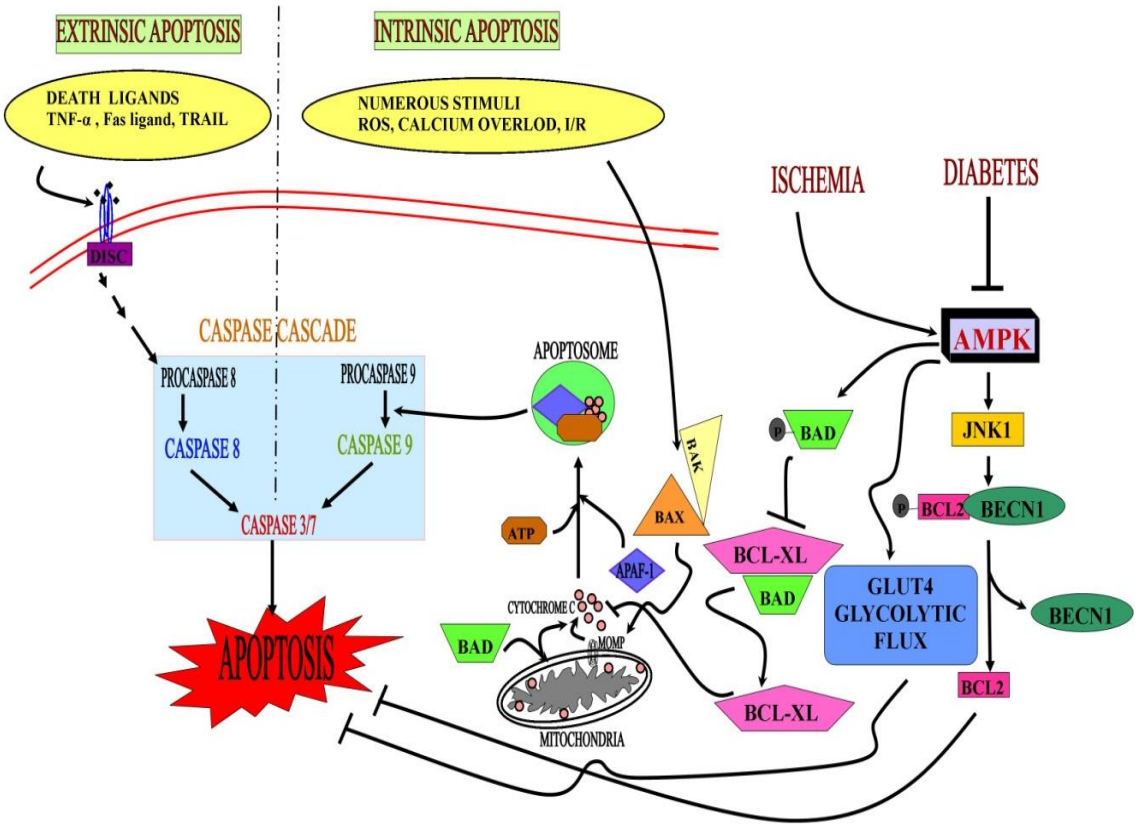


Figure 2.3: Role of AMPK in the regulation of cell death

Death ligands (TNF-α, Fas ligand, TRAIL) activate extrinsic apoptosis by forming DISC and activating caspase 8. ROS, calcium overload, I/R and other various stimuli activate intrinsic apoptosis by activating proapoptotic proteins, BAX and BAK. Both proapoptotic proteins promote the formation of pore and release of apoptogens like cytochrome c, smacDIABLO during mitochondrial outer membrane permeabilization (MOMP). Apoptosome formation (complex of cytochrome c, APAF-1 and ATP) activates procaspase 9 to active caspase 9, thereby activate downstream caspase 3. AMPK exerts antiapoptotic action by activating JNK1-BECN-1-BCL2 pathway and phosphorylating and inactivating Bad (proapoptotic). Phosphorylated Bad restricts its association with BCL-XL (antiapoptotic) and raises its free form concentration and thereby limits apoptosis by preventing cytochrome c release and subsequent caspase activation. During MIRI, AMPK is activated by ischemia and reperfusion which then decrease apoptosis, possibly by improving glucose uptake (raising GLUT4) and glycolytic flux. Apoptosis is more prominent during diabetes as AMPK is suppressed.

Diabetes regulates interplay between cardiac apoptosis and autophagy; triggers apoptotic cell death and diminishes autophagy. AMPK plays a crucial role in the switch between these two cell deaths in diabetic condition. It is reported that reduced AMPK activity is linked to diabetes triggered apoptosis and concomitantly decreased autophagy. Diabetes impairs AMPK activation of MAPK8/JNK1/BCL2 signalling, and subsequent BECN1-BCL2 dissociation thereby promotes apoptosis by suppressing autophagy (Zou & Xie, 2013) (**Figure 2.3**). Additionally, AMPK may target more than one cell death pathway; thus, an in-depth understanding of AMPK role in cross-talk mechanisms of cell death is crucial to moving ahead. Regulation of AMPK seems to have healthy functions in energy stressed myocardium and cardiovascular system, but particular attention should be paid to its detrimental regulation of excessive fatty acid oxidation (Dyck & Lopaschuk, 2006).

Upon stimulation, AMPK phosphorylates different downstream signaling molecules like TSC2 (suppresses protein synthesis by inhibiting mammalian target of rapamycin complex 1) (Inoki et al., 2003), HMG-CoA reductase (inhibits cholesterol synthesis) (Clarke & Hardie, 1990), peroxisome proliferator-activated receptor gamma coactivator (PGC)-1α (activates mitochondrial biogenesis) (Jager et al., 2007) and of predominantly acetyl-CoA carboxylase (ACC) (enables oxidation of fatty acids) (Munday et al., 1988). AMPK stimulation has pleiotropic effects on a multitude of tissues. In liver, AMPK inhibits gluconeogenesis, as well as the synthesis of cholesterol, fatty acid and proteins, while activates uptake of glucose and oxidation of fatty acids (Ruderman et al., 2013). Similarly, in heart, AMPK activates glucose uptake, glycolysis and fatty acid oxidation (Srivastava et al., 2012). Nearly all of the basal functions of peripheral AMPK stimulation would be advantageous for type 2 diabetic patients. It is evident that metabolic syndrome animal models have demonstrated reduced AMPK activity in

muscle (Ruderman & Prentki, 2004). Furthermore, evidence also exists for diminished AMPK activity in adipose (Xu et al., 2012) or skeletal muscle (Bandyopadhyay et al., 2006) of type 2 diabetic or obesity patients. Therefore, the pharmacological manipulation of AMPK has been apparently a potential target for the amelioration of diabetes and its associated cardiac complications.

The AMPK regulation of energy metabolism is extremely significant to cardiac disease, where alterations in energy homeostasis can lead to dysregulation of myocardial contractions and eventually cardiac cell death. AMPK stimulates essential pathways involved in glucose metabolism and its uptake when oxidative metabolism is decreased in the ischemic myocardium. After the commencement of ischemia, decreased cardiac function reduces the requirement of ATP, but AMPK also suppresses energy consuming processes like protein synthesis. Thus AMPK preserves cardiac ATP content during ischemia-reperfusion by modulating energy conserving and generating actions (Russell et al., 2004b). The substantial evidence demonstrated that endogenous activation of AMPK limits the myocardial injury during ischemia-reperfusion studies (Kusmic et al., 2010; Wang et al., 2009).

AMPK signalling has an intricate balance with redox modulation in the vascular milieu. AMPK suppresses ROS generation by inhibiting NADPH oxidase and augment the release of NO by increasing eNOS activity (Fisslthaler & Fleming, 2009). Moreover, AMPK has also been involved in NF-kB-mediated transcription, JNK activation, VCAM-1 expression in endothelial cells that have been subjected to TNF-α, H2O2 or fatty acids exposure, and responsible for decreased monocyte adhesion to the endothelial surface (Hattori et al., 2008; Schulz et al., 2008). AMPK can also manipulate the cellular redox balance by inhibiting high-glucose induced nitration and

prostacyclin synthase by increasing mitochondrial expression of uncoupling protein-2 via stimulation of p38 kinase (Xie et al., 2008).Overall, multiple studies reiterate the notion that AMPK stimulation is susceptible to cellular redox system and the association seems to be unrelated to its putative function of cellular energy homeostasis.

2.5.2. Nuclear factor-erythroid (NF-E) 2-related factor 2 (Nrf2)

Regulation of endogenous oxidant and antioxidant balance under both nonstressed and stressed situations happens majorly at the transcriptional levels, and the Nrf2/ARE signalling plays a critical role in the mediation of cellular redox homeostasis. Nrf2 belongs to Cap "n" collar family of transcription factors, is a major transcriptional regulator of ARE-bearing antioxidants and controls the expression of several genes and functions linked with cell survival and redox balance, including phase I and II detoxification enzymes, free radical scavenging proteins and suppression of inflammation (Kensler et al., 2007), which play an essential role in the pathology of heart diseases (Hybertson et al., 2011). Under nonstressed situations, the Nrf2 function is inhibited by a cytoplasmic protein, Kelch-like ECH-associated protein 1 (Keap1) through interaction with phosphorylation site present on C terminus of Nrf2, and enable it to be ready for ubiquitination and subsequent proteasomal degradation (McMahon et al., 2003). Nrf2, once stabilized, cannot be inhibited by keap 1 and ready to form heterodimers with members of Maf transcription factors. The Nrf2 heterodimer, with the help of nuclear localization sequence, quickly translocates into the nucleus and activates ARE-mediated antioxidant genes, including NADPH quinine oxidoreductase (NQO-1), hemoxygenase-1 (HO-1), catalase, superoxide dismutase (SOD1) and glutathione S-transferase A2 (GSTA2), γ-glutamylcysteine synthase (γ-GCS) and glutathione peroxidase (GPx) (Kensler et al., 2007), which offer first-line defense

against oxidative stress-mediated cardiac damage. SOD facilitates the dismutation of superoxide radical (O_2^-) into hydrogen peroxide (H_2O_2) and oxygen molecule (O_2). The glutathione (GSH)/GPx system plays a significant role in the handling of low-level oxidative stress, by directly scavenging hydroxyl and singlet oxygen radicals and detoxifying lipid peroxides and H_2O_2 (Forman et al., 2009). Catalase transforms the reactive H_2O_2 molecules into nonreactive oxygen and water molecules (Cai, 2005). HO-1 diminished cellular damage through its anti-inflammatory, antioxidant and antiapoptotic effects (Abraham & Kappas, 2005). Thus, Nrf2, by inducing transcriptional expression of a battery of antioxidant genes, can improve cellular antioxidant defence system, thereby increasing the cellular capacity to nullify the toxic effects of oxidative stress (**Figure 2.4**). Besides controlling the global antioxidant balance, Nrf2 also regulates the transcription factors involved in mitochondrial function and anti-inflammatory genes. Therefore, Nrf2 signalling pathway represents a promising target in treating oxidative stress associated diseases like insulin resistance and a broad spectrum of diabetic complications.

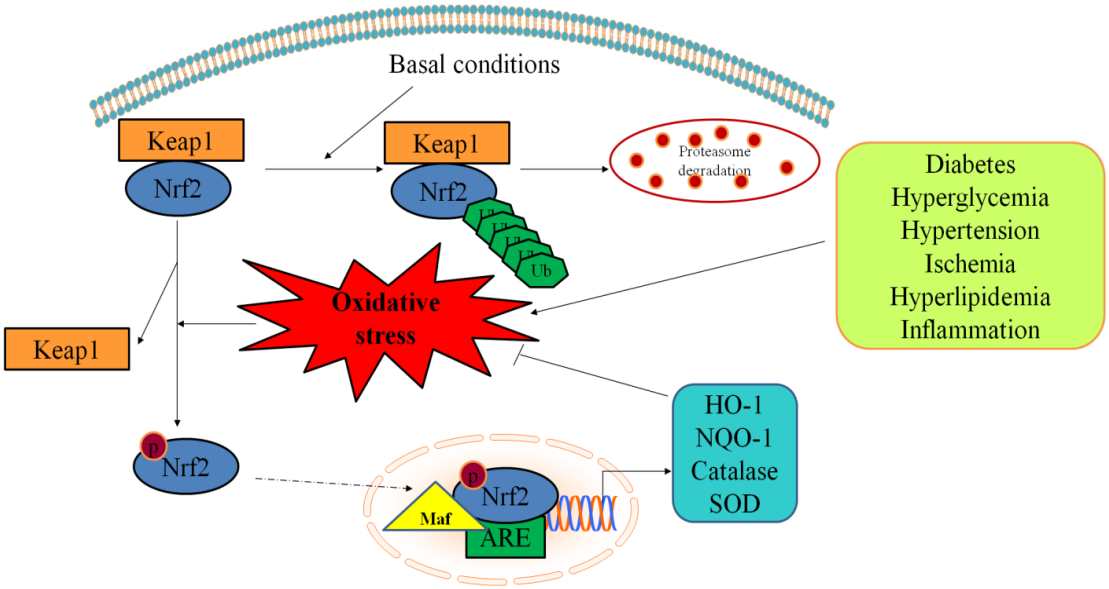


Figure 2.4: Role of Nrf2 signalling in oxidative stress.

Under basal conditions, Keap1 binds to Nfr2 and promotes phosphorylation on C terminus of Nrf2, and enable it to be ready for ubiquitination and subsequent proteasomal degradation. Under disease conditions like diabetes, hypertension where excessive oxidative stress prevails, Nrf2 dissociates from Keap1 and binds to Maf and ARE to activate transcription of antioxidant genes such as catalase, SOD, NQO-1 and HO-1, which in turn inhibit the oxidative stress.

Diabetes alters the expression pattern of Nrf2 and thereby antioxidant defence system. Tan et al. (2011) demonstrated that expression of the Nrf2 protein was slightly upregulated in two-month diabetic mice heart but markedly downregulated in the hearts of five-month diabetic mice (Tan et al., 2011). These results suggest that overexpression of Nrf2 was a homeostatic mechanism to prevent diabetic damages at the initial stage of diabetes; however, at the later stages, myocardial antioxidant defence system was so severely weakened and leading to diminished myocardial Nrf2 expression (Li et al., 2012). Furthermore, the absence of Nrf2 has been associated with aggravation of both type 1 and 2 diabetic conditions (Aleksunes et al., 2010; Bitar & Al-Mulla, 2011).

Substantial evidence indicated that Nrf2 offers protection against heart failure and pathological cardiac hypertrophy. Nrf2 overexpression in mouse hearts decreased ROS generation and attenuated transverse aortic constriction (pressure overload)-induced hypertrophy of cardiomyocytes and cardiac fibroblasts (Li et al., 2009). This cardioprotective potential of Nrf2 in heart failure and myocardial remodelling can be linked to Nox4 isoform (Brewer et al., 2011), which is essential for regulation of redox balance in cardiomyocytes and is plays a crucial role in the development of pressure overload-induced mitochondrial oxidative stress (Kuroda et al., 2010). In murine models of type 2 diabetes and heart failure, oxidative stress downregulates the cardiac expression of Nrf2 and decreases glucose consumption, leading to the development of insulin resistance (Tan et al., 2011). Furthermore, Nrf2 expression was downregulated in the later stage of diabetic cardiomyopathic mice model (Tan et al., 2011). Conversely, upregulation of Nrf2 suppresses ROS and aberrant cardiac hypertrophy via extracellular signal-related kinase (Erk) signalling, which activates Nrf2 during stressed situations where enhanced oxidative stress prevails (Li et al., 2009). Several experiments employing pharmacologic Nrf2 stimulators have implicated in cardioprotection through antioxidant properties (Ashrafian et al., 2012; Xing et al., 2012).

Simulated ischemia-reperfusion (10 hrs hypoxia, followed by 16 hrs normoxia) resulted in a marked rise of endogenous ROS levels in rat cardiac H9C2 cells, while phase II antioxidant enzyme inducer D3T treatment significantly increased mRNA and protein content of Nrf2, as well as reduced intracellular ROS levels under the same conditions (Cao et al., 2006). This study signified the importance of Nrf2 in limiting intracellular concentrations of ROS following ischemia-reperfusion (Cao et al., 2006). Intriguingly, acute stimulation of Nrf2 by ischemic preconditioning has been shown to protect

myocardium from ischemia-reperfusion injury (Gurusamy et al., 2007). Zhang et al. demonstrated that ischemic preconditioning decreased cardiac infarct size and tissue malondialdehyde content through stimulation of protein kinase C and nuclear translocation of Nrf2, thereby inducing MnSOD and HO-1 (Zhang et al., 2013b). Therefore, the beneficial effects of activation of Nrf2 pathways can be linked to decreasing in oxidative stress, inflammation and apoptosis (Zhao et al., 2013).

2.5.3. Hemoxygenase-1 (HO-1)

ROS and heavy metals can able to induce HO-1, which catalyzes the breakdown of heme molecule into carbon monoxide, ferrous iron (Fe_2^-), and biliverdin, the latter being subsequently transformed into bilirubin (Ponka, 1999). HO-1 and its products regulate several basal functions, as well as play a role in the prevention of pathophysiology of cardiovascular diseases associated with oxidative stress (Victor & Rocha, 2007) (**Figure 2.5**). HO-1 seems to have a role in diminishing hyperglycemia and TNF-α-induced apoptosis (Iori et al., 2008). HO-1 gene overexpression in rat heart by adenovirus-mediated transfection process resulted in decreased cardiac infarct size, accompanied by reduced levels of lipid peroxidation, IL-1β, and proapoptotic Bax, with a simultaneous rise in Bcl-2 levels (Melo et al., 2002).

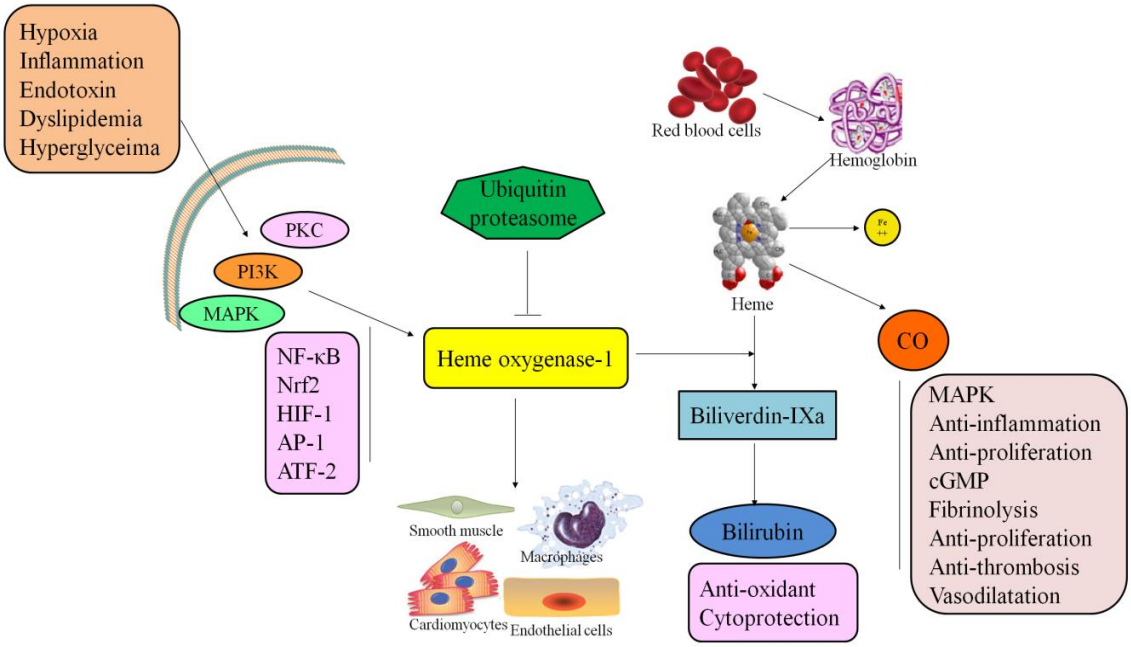


Figure 2.5: Role of HO-1 in diabetic cardiomyopathy.

The expression of HO-1 is induced by numerous stimuli, including oxidants, cytokines, hypoxia, endotoxin, hyperglycemia and inflammation via several distinct signalling pathways. HO-1 catalyzes the enzymatic degradation of heme of red blood cells to release carbon monoxide (CO), iron and biliverdin, with the latter being subsequently converted to bilirubin by biliverdin reductase. Bilirubin has a potential cytoprotective and antioxidant action. Activation of HO-1 confers cardioprotection via the diverse effects of bilirubin and CO on macrophages, cardiomyocytes and vascular cells. The ubiquitin-protease system degrades the HO-1 protein. cGMP: cyclic guanosine monophosphate; MAPK: mitogen-activated protein kinase; PKC: protein kinase-C; PI3K: phosphatidylinositol-3-kinase; NF-κB: nuclear factor- κB; HIF-1: hypoxia-inducible factor-1; Nrf2: Nuclear factor-erythroid (NF-E) 2-related factor 2; AP-1: activator protein-1; ATF-2: activating transcription factor-2.

The significance of HO-1 in the regulation of cardiac homeostasis was first identified in an experiment demonstrating that cardiac expression of HO-1 is elevated when challenged to hyperthermia (Ewing et al., 1994). A follow-up study reported that HO-1 expression is upregulated in the porcine heart in response to ischemia-reperfusion, indicating a promising role of HO-1 in combating cardiac pathophysiological stress situations (Sharma et al., 1996). Genetic loss-of-function experiments using cardiac-specific HO-1 deficient mice in contrast to wild-type mice, demonstrated that hypoxia

caused severe infarction and right ventricular dilatation in HO-1 null mice (Yet et al., 1999). Further, HO-1 deficiency aggravated ischemia-reperfusion-induced cardiac injury (Liu et al., 2005). In a gain-of-function study employing transgenic mice, overexpression of cardiac-specific HO-1 decreased cardiac inflammatory cell infiltration and infarct size, following ischemia-reperfusion challenge, further suggesting the beneficial role of HO-1 against cardiovascular complications like myocardial infarction (Yet et al., 2001). Similarly, upregulation of HO-1expression reduced apoptosis and ameliorates neovascularization in the heart failure model (Wang et al., 2010). Furthermore, increased expression of HO-1 in cardiomyocytes prevented ischemia-reperfusion injury-induced cardiac oxidative stress and inflammation (Yet et al., 2001).

HO-1 is a well-known regulator of the inflammatory response. HO-1 knockout mice, upon exposure to lipopolysaccharides, have exhibited greater end-organ damage and diminished survival rate when compared to wild-type mice (Wiesel et al., 2000). In endothelial cells, HO-1 and bilirubin decreased TNF-α induced overexpression of E-selectin and VCAM-1 by suppressing NF-κB stimulation (Soares et al., 2004). Similarly, upregulation of HO-1 or its products has led to protective antiproliferative and anti-inflammatory effects in in-vivo models of in-stent restenosis, vascular injury and transplant arteriosclerosis by reducing apoptosis, leukocyte infiltration, NF-κB stimulation and pro-inflammatory cytokine expression (Bouche et al., 2002; Du et al., 2007). Besides, HO-1 upregulation in the heart reduces IL-1β expression, lipid peroxidation, apoptosis and cardiac infarct size (Melo et al., 2002). Therefore, HO-1 exerts beneficial effects on cardiovascular diseases through anti-inflammatory, antioxidant and antiapoptotic effects.

2.6. Need and Scope of Herbal Medicine for Diabetic Cardiomyopathy

Stringent glycemic and blood pressure control, in addition to aggressive treatment of lipid abnormalities, appears to be essential for the treatment of diabetic cardiomyopathy and other cardiac pathologies in diabetic patients. Hence, there is a need for the search of potential drug candidates for the benefit of long-term health outcomes in diabetic populations. Recently, herbal/natural products have attracted the attention of scientific community as adjuvant drugs or alternate choice for treating diabetes-associated cardiovascular complications. Abundant evidence has highlighted that many natural products exert antidiabetic activity through antioxidant and anti-inflammatory properties (Giovannini et al., 2016). Since oxidative stress and inflammation responsible for the diabetes-induced deleterious effects (Domingueti et al., 2016), herbal/natural products coupled with high safety profile, antioxidant and anti-inflammatory properties, remain potential candidates to overcome diabetic cardiomyopathy. However, further research is warranted to reveal the therapeutic efficacy in the management of diabetic cardiac pathologies.

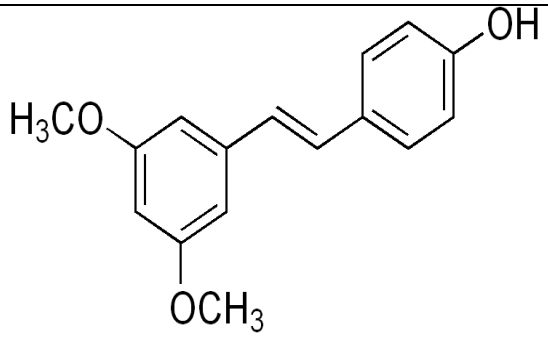
2.7. Pterostilbene

2.7.1. Structure and history

Pterostilbene (PT), or 3,5-dimethoxy-4'-hydroxystilbene (molecular weight: 256.3), is a phytoalexin (Langcake & Pryce, 1977) and naturally derived non-flavonoid polyphenol compound with a structure similar to that of resveratrol (3,5,4'-trihydroxystilbene) (**Table 2.2**). PT is a lipid-soluble compound that exists in cis and trans forms, with the trans form being most abundant. It was first isolated from the heartwood of red sandalwood (*Pterocarpus santalinus*) (Seshadri, 1972) and later identified in grapevines

(*Vitis vinifera*) (Langcake et al., 1979) and blueberries (Rimando et al., 2004). Interestingly, PT was identified as the primary phenolic compound in the wood of Indian kino (Pterocarpus marsupium), and drakshasava has been used by Ayurvedic practitioners in the treatment of diabetes (Manickam et al., 1997), cardiovascular and related problems (Paul et al., 1999) since ancient times. In 2002, Rimando and colleagues reported that PT acts as a cancer chemopreventive agent, due to its ability to scavenge peroxyl radicals (Rimando et al., 2002). Research interest in PT increased after 2011 when it was shown to have antiproliferative effects in cultured cells at lower concentrations than resveratrol (McCormack et al., 2011). Meanwhile, antiinflammatory (Remsberg et al., 2008), antiobesity (Rimando et al., 2005) and antioxidant (Remsberg et al., 2008; Rimando et al., 2002) properties have also been reported. More recently, clinical trials have also been conducted to evaluate the potential of PT in treating or preventing cardiac diseases (Riche et al., 2014b). Therefore, PT has become a highly valuable natural bioactive phytonutrient with potential therapeutic applications and market prospects.

Table 2.2: PT structure and formula

Structure	
Molecular weight	256.299 g/mol
Moleuclar formula	$C_{16}H_{16}O_3$
Other names	Dimethoxy resveratrol; 3',5'-Dimethoxy-4-stilbenol; 3,5-Dimethoxy-4'-hydroxy-trans-stilbene.

2.7.2. Sources of PT

Pterostilbene is synthesized in plants as a secondary metabolite in response to environmental stress or fungal infections (Pezet & Pont, 1990). The concentration of PT in various foodstuffs is summarized in **Table 2.3.**

Table 2.3: PT content in certain natural foods

Foodstuff	Concentration range	References
Blueberries	9.9-15.1 µg/kg of fresh weight 15 µg/100 g of weight	(Aiyer et al., 2012) (Rimando et al., 2004)
Fungal infected grapes	0.2-4.7 µg/g of fresh weight of skin	(Adrian et al., 2000)
Healthy grape berries var. Gamay	14-74 ng/g fresh berries	(Pezet & Pont, 1988)
Healthy grape berries var. Pinot Noir	120-530 ng/g fresh berries	(Pezet & Pont, 1988)
Vaccinium ashei (rabbit eye blueberry)	99-151 ng/g dry sample	(Rimando et al., 2004)
Vaccinium stamineum (deerberry)	520 ng/g dry sample	(Rimando et al., 2004)

2.7.3. Effects of PT on Metabolic and Cardiovascular Diseases

2.7.3.1. Antidiabetic mechanism of PT

Recent preclinical and clinical evidence suggests that PT also exerts a strong influence on glucose homeostasis. Pterostilbene has been shown to decreased plasma glucose levels and increased plasma insulin levels significantly in diabetic animals (Amarnath Satheesh & Pari, 2006; Manickam et al., 1997; Pari & Satheesh, 2006). Oral administration 40 mg/kg PT to diabetic rats for six weeks decreased hepatic gluconeogenic enzymes glucose-6-phosphatase and fructose-1,6-bisphosphatase and increased the hepatic glycolytic enzyme hexokinase (Pari & Satheesh, 2006). Moreover, PT treatment enhanced the peripheral utilization of glucose and restored the deficient

hepatic glycogen stores mediated by increases in the activity of both skeletal and hepatic hexokinase and hepatic phosphofructokinase enzymes in diabetic rats (Grover et al., 2002). Furthermore, PT treatment prevented a significant elevation in glycosylated haemoglobin (HbA1c) and increased the total haemoglobin level through improved glycemic control in diabetic rats (Pari & Satheesh, 2006). In another study, PT (15 mg/kg/day) decreased insulin resistance in obesogenic fed rats by increasing glucose transporter 4 protein expression in skeletal muscle, mediated through activation of Akt via stimulation of cardiotrophin-1 (Gomez-Zorita et al., 2015).

Discrepant results have been observed with PT in the context of glucose-regulating enzymes under different settings, such as type 2 diabetes and insulin resistance, and also at different dose levels. The possible explanation for this is that gluconeogenic enzyme (e.g. glucose-6-phosphatase) are not involved in amelioration of insulin resistance by PT in an obesogenic fed rat model (Gomez-Zorita et al., 2015) whereas PT downregulated the same enzyme activity in streptozotocin type 2 diabetic rats (Pari & Satheesh, 2006), irrespective of PT dose. However, PT improves glycemic control in insulin resistance by influencing glycolytic enzymes in both the liver and skeletal muscle, alongside skeletal muscle glucose uptake (Gomez-Zorita et al., 2015). A lower dose of PT (15 mg/kg/day) produced more beneficial antidiabetic activity than a higher dose (30 mg/kg/day) in an obesogenic diet-induced insulin resistance rat model. This phenomenon is because low dose PT increased hepatic glucokinase activity and skeletal muscle glucose uptake whereas the higher dose influenced skeletal muscle glucose uptake only, rather than hepatic glucokinase activity (Gomez-Zorita et al., 2015).

Besides controlling hyperglycemia efficiently, another proposed mechanism by which PT confers protective effects against diabetic complications is through its ability to

reduce oxidative stress, which plays a critical role in aberrant glucose regulation (Amarnath Satheesh & Pari, 2006; Pari & Satheesh, 2006). PT (40 mg/kg) treatment rendered renal and hepatic cells of diabetic rats more resistant to the detrimental effects of oxidative stress by increasing the levels of antioxidant enzymes like GSH, GST, SOD, GPx and catalase (Amarnath Satheesh & Pari, 2006). These results were suggesting that PT improved tissue resilience against oxidative stress and prevented end-organ damage via its antioxidant capability (Amarnath Satheesh & Pari, 2006).

Whether the antidiabetic properties of PT extend to humans is still unknown. An aqueous extract of heartwood of Pterocarpus marsupium (3 g) was tested clinically and found to be effective in lowering blood sugar in non-insulin–dependent diabetes mellitus patients (1998). The antioxidant activity of blueberries was investigated by Nemes-Nagy et al. (2008) in children with diabetes mellitus. They found that two months treatment with blueberry extract significantly enhanced the antioxidant capacity of red blood cells by increasing the activity of SOD and GPx and decreasing the levels of HbA1c (Nemes-Nagy et al., 2008). This outcome can likely be attributed to the antioxidant ability of PT; however, further experiments are essential to determine the active compound in blueberries and explore a possible relationship with PT. A recent clinical trial conducted by a Mississippi hospital demonstrated that PT treatment reduced plasma free radical levels in a randomized, double-blind, placebo-controlled trial (Riche et al., 2014b).

2.7.3.2. Antihyperlipidemic mechanism of PT

Recently, attempts have been made to evaluate the antiobesity properties of PT (Hsu et al., 2012; Rimando et al., 2005). The lipid reducing impact of PT is credited mainly to its induction of PPAR-α, a familiar target for dyslipidemia therapy (Fruchart et al., 2003). PPAR-α is primarily implicated in the metabolism of fatty acids and lipids through the transcriptional activation of fatty acid β-oxidative genes, thereby displaying pleiotropic impacts in cardiac, hepatic and skeletal muscle tissues (Fruchart et al., 2003; Rimando et al., 2005). In H4IIEC3 cell line (rat liver cell line), PT (at 100 μM and 300 μM) exhibited higher PPAR-α induction (about 8 to 14-fold increase compared to control) than 100 μM ciprofibrate, a standard hypolipidemic drug (a five-fold increase compared to control) (Rimando et al., 2005). Further, PT (25 ppm) fed hypercholesterolemic hamsters demonstrated a 14% reduction in plasma glucose and a 29% reduction in plasma low-density lipoprotein (LDL) cholesterol levels, but a 7% increase in plasma high-density lipoprotein (HDL) cholesterol levels relative to control animals, through induction of PPAR-α (Rimando et al., 2005). In addition, PT (5μM and 10 μM) administration downregulated leptin, PPAR-γ, CCAAT/enhancer binding protein (C/EBP)-α, resistin, and fatty acid synthase (FAS), and upregulated adiponectin in an adipocyte cell line (3T3L1) model (Hsu et al., 2012). Pan and colleagues (2008) revealed that a large number of genes involved in lipid metabolism were affected when *Saccharomyces cerevisiae* was exposed to PT (IC50 = 70 μM) for one generation (3 h) (Pan et al., 2008b). Pterostilbene upregulated the genes for enzymes associated with fatty acid β-oxidation. Additionally, PT also enhanced the expression of various genes concerned with sterol, phospholipid and sphingolipid metabolism. Collectively, these findings suggest that PT mediates its antidyslipidemic activity through regulation of molecular pathways of lipid metabolism by modulating the PPAR-α gene.

Moreover, PT suppresses lipogenesis by activating AMPK in human prostate cancer cells (Lin et al., 2012) and adipose cells (Gómez-Zorita et al., 2014) at variable concentrations 80 μM and 1 μM, respectively. PT has also demonstrated antiobesity effects in rats at 15 mg/kg/day, resulting from reduced lipogenesis (decreased acetyl-CoA carboxylase activity mediated by AMPK stimulation) in adipose tissue and increased fatty acid oxidation (increased carnitine palmitoyltransferase I (CPT-1)A and acyl-CoA oxidase activity) in the liver. In addition, PT decreased total adipose mass in rats; however, a more significant effect (22.9 vs 15.1%) was observed at a higher dose (30 mg/kg) than the lower dose (15mg/kg). The higher dose of PT decreased both subcutaneous and internal adipose depots (mesenteric and perirenal), whereas the lower dose of PT mainly affected the subcutaneous depots (Gómez-Zorita et al., 2014). Besides, PT (5 μM) has also been demonstrated to reduce several inflammatory markers, such as IL-6, in 3T3-L1 adipocytes after induction of inflammation by TNF-α (Hsu et al., 2013). In insulin-resistant rats, PT enhanced fatty acid oxidation in the gastrocnemius muscle due to induction of greater mitochondrial oxidative capacity and mitochondriogenesis by increasing CPT-1B, citrate synthase, mitochondrial transcription factor A (a marker of mitochondriogenesis) and cytochrome c oxidase subunit II activities (Gomez-Zorita et al., 2015).

Riche and colleagues (Riche et al., 2014a) evaluated the antihyperlipidemic effects of PT in 80 subjects from a mixed population of Caucasian, African American and Asian individuals. Patients were randomized in a 2 X 2 block design into one of four groups: 50mg PT twice daily (low dose), 125 mg PT twice daily (high dose), 50 mg PT + 100 mg grape extract twice daily (low dose + grape extract), or matching placebo by mouth twice daily, for 52 days (This trial is registered with Clinicaltrials.gov NCT01267227). In contrast to the preclinical animal studies, both PT groups showed increased LDL

cholesterol levels and no changes in triglyceride or HDL levels (Riche et al., 2014a). The reason for PT augmentation of LDL levels remains uncertain, but possible causative factors, such as cross selectivity with PPAR-γ and enhanced catabolism of triglyceride-rich lipoproteins, might explain these findings. However, further studies should be carried out to explore the effects of PT on cholesterol levels in humans. PT significantly reduced body weight in specific subgroups. Moreover, there were several shortcomings with this trial, including its single centred design, small sample size, and acute duration (Riche et al., 2014a). Ultimately, the clinical potential of PT for treating cardiovascular ailments may be attributable to its lipid-lowering activity. Further investigation is warranted to ascertain the beneficial effects of PT in diabetic patient populations, in addition to healthy controls.

2.7.3.3. Antiatherosclerotic mechanism of PT

Vascular smooth muscle cells (VSMCs) are regarded as the chief cellular elements of the arterial blood vessel wall, and abnormal proliferation of VSMCs plays a central role in the pathogenesis of atherosclerosis, leading to heart diseases like hypertension (Ross, 1990; Schwartz, 1997). Inhibition of VSMC proliferation is essential in the treatment of cardiovascular disease. A study performed by Park et al. (2010) suggested that PT exerts a remarkable inhibitory effect on both DNA synthesis and proliferation of rat aortic VSMCs, with IC50 values of 1.08 ± 0.02 and 1.53 ± 0.04, respectively (Park al., 2010). In addition, treatment with PT at 1, 3 and 5 μM resulted in decreased expression of cell cycle regulating factors such as Cdk2, Cdk4, cyclin D1, cyclin E, retinoblastoma proteins and PCNA (all of which contribute to atherosclerosis by facilitating the aberrant growth of VSMC) in a concentration-dependent manner (Park et al., 2010).

Thus, the antiproliferative effects of PT may, therefore, confer a defence against atherosclerosis and subsequent complications of stenosis.

Pterostilbene has been shown to counteract oxidized LDL (oxLDL) induced pro-atherosclerosis through its modulating effect on cell death programs such as apoptosis and autophagy. In independent experiments performed by Zhang et al., (2012, 2013) PT (1 μM) prevented detrimental apoptosis and stimulated autophagy in vascular endothelial cells (VECs), thereby confronting the oxLDL engendered pro-atherosclerosis effect in VECs (Zhang et al., 2013a; Zhang et al., 2012). Treatment with PT enhances autophagy through stimulation of AMPK-mammalian target of rapamycin signalling via a brisk elevation in intracellular calcium levels, and subsequent CaMKKβ stimulation (Zhang et al., 2013a).

oxLDL-induced apoptosis is considered to have a central role in the development of atherosclerosis, both in the early stages of lesion formation and later during disease development (Salvayre et al., 2002). Pterostilbene diminished several apoptotic mediators, such as p53 accumulation and the activity of several caspase enzymes (including caspase 9 & 3), whereas PT amplified MMP and cytochrome c release in oxLDL treated human umbilical vein endothelial cells by suppressing lectin-like oxLDL receptor-1 (LOX-1) expression (Zhang et al., 2012). Besides, PT pretreatment attenuated both oxidative stress and inflammation by decreasing ROS and NF-κB activation, respectively (Zhang et al., 2012). Thus, PT has been shown to exert antiatherosclerotic action by modulating LOX-1, NF-κB, and the antioxidant enzymes SOD and catalase, providing protection against inflammation and oxidation.

2.7.3.4. Infarct sparing mechanism of PT

A few studies have demonstrated that blueberries, and PT alike, are protective against cardiovascular disease, perhaps because they stimulate antioxidative enzymes. A previous study conducted by Ahmet et al. (2009) suggested that consuming a blueberry-enriched diet enhanced myocardial tolerance to ischemic injury. Three-month blueberry supplementation (20 g/kg) reduced the myocardial infarction (MI) size by 22% in a rat model of coronary artery ligation compared to rats provided a regular diet (Ahmet et al., 2009). Furthermore, blueberry-enriched diet increased cardiomyocyte survival by elevating the mitochondrial permeability transition ROS threshold, thereby attenuating necro-apoptosis and inflammation in ischemic cells (Ahmet et al., 2009).

Pterostilbene has proven to be effective at protecting rat hearts against ischemia/reperfusion injury. In a recent study, intravenous administration of PT (100 μmol/l) 5 min before reperfusion to rats undergoing 30 min of ischemia/120 min of reperfusion resulted in reduced myeloperoxidase levels, both serum and myocardial TNF-α production and a reduction in infarct size and apoptotic index (Wang et al., 2015a). This effect could be at least partially related to the anti-inflammatory activity of PT via inhibiting toll-like receptor 4/NF-κB signalling pathway. Inhibitors of NOS and cyclic guanosine monophosphate (cGMP) block the protective effects of PT in rats, demonstrating that this mechanism could be relevant in vivo. Nitric oxide and cGMP signalling pathways play a pivotal role in the protective effects of PT (Lv et al., 2015; Wang et al., 2015a). Still, further studies are required to elucidate the protective role of PT against myocardial infarction in the proximity of diabetes, metabolic syndrome and obesity.

2.7.3.5. Antihypertensive mechanism of PT

The cardiovascular protection conferred by red wine is thought to be attributed to the presence of polyphenols, such as PT and resveratrol (Das et al., 1999). Potential blood pressure (BP) lowering mechanisms, such as diminution of angiotensin-converting enzyme, triggering of antioxidative pathways, and augmentation of vascular endothelial NOS, have been reported for PT (McCormack & McFadden, 2013; Shaul, 2002). Pterostilbene decreases BP in adults when administered at a dose of 250 mg/day (Riche et al., 2014b). Recent clinical trials explored the efficacy of PT in lowering BP demonstrated that PT reduced both systolic and diastolic BP without any change in the overall Atherosclerotic Cardiovascular Risk in adults (Riche et al., 2014b). Intriguingly, the BP reduction observed with PT is comparable to that seen with selective PPAR-γ agonists (Negro et al., 2005). Furthermore, PT (250 mg/day) has been shown to be safe in normotensive patients, as it did not produce hypotension or symptomatic orthostasis (Riche et al., 2014b). Therefore, PT could be an attractive therapeutic option in the management of hypertension. However, further clinical trials with long durations should be carried out to scrutinize the potential of PT as a therapeutic alternative for delaying the transformation from prehypertension to hypertension.

2.7.3.6. Antihematologic action of PT

Beneficial effects of PT on macrophages are due to its ability to downregulate the expression of inflammatory iNOS and COX2 genes by inhibiting NF-κB stimulation, thereby suppressing the production of proinflammatory cytokines in response to inflammatory stimuli (Pan et al., 2008a; Qureshi et al., 2012). A study conducted by Numico Research, The Netherlands team, found that PT from Pterocarpus marsupium inhibited prostaglandin E_2 (PGE_2) production from lipopolysaccharide-stimulated

human blood cells. In healthy human volunteers, the plant extract did not decrease PGE_2 production, because PT levels in serum were below the active concentrations observed in vitro (Hougee et al., 2005b). Further, PT is effective in inhibiting collagen-induced platelet aggregation and stimulating platelet NO production by inhibiting platelet ROS generation (Messina et al., 2015). These results suggest that PT is a potential antithrombotic agent.

In-vitro and in-vivo tests were conducted by Youdim and colleagues to determine the antioxidant capability of blueberry polyphenols in vulnerable red blood cells (RBCs). These studies revealed that dietary blueberry supplementation to rats offered protection of RBCs against free radical generation after hydrogen peroxide exposure (Youdim et al., 2000). Further, Mikstacka and colleagues demonstrated that PT treatment prevented the 2,2-azobis 2-amidinopropane dihydrochloride prompted RBC hemolysis as well as diminution of GSH levels (Mikstacka et al., 2010). Besides, PT treatment was found to counteract lipid peroxidation induced by hydrogen peroxide, thereby reducing autoxidation in RBCs (Stocks & Dormandy, 1971). It has been hypothesized that blueberries, and their constituent PT, offer protection to RBCs against oxidative stress by scavenging hydrogen peroxide and by increasing antioxidant activity.

Thus, PT, which is present in grapes, blueberries, and red wine, may contribute to lowering the incidence of cardiovascular disease and may offer cardioprotection through several mechanisms, as discussed above.

2.7.4. Safety

Recent studies have investigated the safety profile of PT. Most of the human and animal data suggest that PT does not have significant toxic effects. For example, no toxic effects or mortality were observed in mice given oral supplementation of PT over the dose range of 0, 30, 300, and 3000 mg/kg/day for four weeks (Ruiz et al., 2009). Moreover, PT fed mice showed increased RBC counts and hematocrit relative to control mice. Further, biochemical and histopathological observations showed no serious modifications in organ weight or clinical signs of disease over the dose range of PT (Ruiz et al., 2009). The toxicity of PT was also assessed in mice after i.v. administration of 30 mg/kg/day for 23 days. Even at this high dose, PT was pharmacologically safe as its administration was accompanied by no systemic or organ related toxicity. In humans, Riche et al. (2013) found no toxic effects of PT after administration of doses up to 250 mg per day (Riche et al., 2013). Biochemical analysis revealed no adverse drug reactions related to liver, kidney and blood glucose levels when 125 mg PT was administered twice daily (Riche et al., 2013). In addition, PT (pTeroPure) recently attained safe status (www.fda.gov). Despite these findings, more rigorous experiments are required before dietary/therapeutic dosages of PT can be standardized for different therapies.
