Université de Montréal

Role of Sirtuin-1 in the Pathogenesis of Hypertension in Spontaneously Hypertensive Rats: Molecular Mechanisms

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Rôle de la Sirtuine- 1 dans la pathogenèse de l'hypertension chez les rats spontanément hypertendus: Mécanismes moléculaires

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Résumé

Il a été démontré que la sirtuine 1 (Sirt-1), une histone désacétylase de classe III, est surexprimée dans le cœur des rats spontanément hypertendus (SHR). Nous avons récemment montré que les cellules musculaires lisses vasculaires (CMLV) des SHR présentent une expression accrue de Sirt-1 par rapport aux rats Wistar Kyoto (WKY) de même âge qui contribue à l'augmentation de la régulation de la protéine Giα impliquée dans la pathogenèse de l'hypertension. La présente étude a été effectuée pour étudier le rôle de l'augmentation de l'expression de la Sirt-1 dans la pathogenèse de l'hypertension chez les SHR et pour explorer les mécanismes moléculaires impliqués dans cette réponse. Dans cette étude, un inhibiteur sélectif de la Sirt-1, EX-527 (5 mg/kg de poids corporel), a été injecté par voie intrapéritonéale chez des rats SHR adultes de 8 semaines et des rats WKY de même âge, deux fois par semaine pendant 3 semaines. La pression artérielle (PA) et la fréquence cardiaque ont été mesurées deux fois par semaine par la méthode non invasive du brassard autour de la queue. Le traitement avec l'inhibiteur spécifique de la Sirt-1, l'EX-527, a atténué les augmentations de PA (de 76 mmHg) et de fréquence cardiaque chez les rats SHR. La surexpression de Sirt-1 et des protéines Giα dans le cœur, les CMLV et l'aorte a été atténuée au niveau des contrôles par l'inhibiteur de la Sirt-1. L'inhibition de la Sirt-1 a également atténué les niveaux accrus des anions superoxydes, l'activité de la NADPH oxydase et la surexpression des sous-unités de la NADPH oxydase ; les protéines Nox2, Nox4 et P47phox dans les CMLV isolées des SHR traités par l'EX-527. De plus, les niveaux réduits du monoxyde d'azote synthase endothélial (eNOS) et du monoxyde d'azote (NO) et les niveaux accrus de la peroxynitrite ($ONOO^-$) dans les CMLV des SHR ont également été rétablis à des niveaux contrôles par l'inhibiteur de la Sirt-1. Ces résultats suggèrent que l'inhibition de la surexpression de la Sirt-1, en diminuant les niveaux accrus des protéines Giα et du stress nitro-oxydant, atténue la PA élevée chez les rats SHR. Il est donc possible de suggérer que les inhibiteurs de la Sirt-1 puissent être utilisés comme des agents thérapeutiques dans le traitement des complications cardiovasculaires associées à l'hypertension.

Mots-clés: Sirtuine 1, EX-527, Protéine Giα, SHR, CMLV, Hypertension.

Abstract

Sirtuin-1 (Sirt-1), class III histone deacetylase, has been shown to be overexpressed in hearts from spontaneously hypertensive rats (SHR). We recently showed that vascular smooth muscle cells (VSMC) from SHR exhibit enhanced expression of Sirt-1 as compared to age-matched Wistar Kyoto (WKY) rats, which contributes to the upregulation of Giα protein implicated in the pathogenesis of hypertension. The present study was undertaken to investigate the role of upregulated Sirt-1 expression in the pathogenesis of hypertension in SHR and to explore the underlying molecular mechanisms involved in this response. For this study, a selective inhibitor of Sirt-1, EX-527 (5mg/kg of body weight), was injected intraperitoneally into 8-week-old adult SHR and age-matched WKY rats twice per week for 3 weeks. The blood pressure (BP) and heart rate was measured twice a week by the CODA™ non-invasive tail cuff method. Treatment of SHR with Sirt-1-specific inhibitor, EX-527, attenuated high BP by 76 mmHg and inhibited the augmented heart rate. The overexpression of Sirt-1 and Giα proteins in heart, VSMC and aorta was attenuated to the control levels by Sirt-1 inhibitor. Inhibition of Sirt-1 also attenuated the enhanced levels of superoxide anion, NADPH oxidase activity and the overexpression of NADPH oxidase subunits; Nox2, Nox4 and P47phox proteins in VSMC isolated from EX-527-treated SHR. Furthermore, the decreased levels of endothelial nitric oxide synthase (eNOS) and nitric oxide (NO) and increased levels of peroxynitrite ($ONOO^-$) in VSMC from SHR were also restored to control levels by Sirt-1 inhibitor. These results suggest that the inhibition of overexpression of Sirt-1 through decreasing the enhanced levels of Giα proteins and nitro-oxidative stress attenuates the high BP in SHR. It may thus be suggested that inhibitors of Sirt-1 may have the potential to be used as therapeutic agents in the treatment of cardiovascular complications associated with hypertension.

Key Words: Sirtuin-1, EX-527, Giα protein, SHR, VSMC, Hypertension.

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List of Abbreviations

AC	Adenylyl Cyclase
ACE	Angiotensin-converting Enzyme
Ang II	Angiotensin II
ANP	Atrial Natriuretic Peptide
ATP	Adenosine Triphosphate
BH4	Tetrahydrobiopterin
BP	Blood Pressure
cAMP	Cyclic Adenosine Monophosphate
cGMP	3',5'-Cyclic Guanosine Monophosphate
CO	Cardiac Output
CVDs	Cardiovascular Diseases
DOCA	Deoxycorticosterone Acetate
eNOS	Endothelial Nitric Oxide Synthase
EC	Endothelial Cells
ET-1	Endothelin 1
ET_A	Endothelin receptor Type A
ET_B	Endothelin receptor Type B
GDP	Guanosine diphosphate
Gi	Inhibitory G protein
GPCR	G Protein-coupled Receptor
G protein	Guanine Nucleotide-binding Protein
Gs	Stimulatory G protein
GTP	Guanosine triphosphate
H_2O_2	Hydrogen peroxide
HDAC	Histone deacetylase
JNK1	c-Jun N-terminal kinase 1
L-NAME	Nω-Nitro-L-Arginine Methyl Ester
MAP	Mean Arterial Pressure
MAPKs	Mitogen-activated Protein Kinases
Mm Hg	Millimeters Mercury
MnSOD	Manganese Superoxide Dismutase
NAD^+	Nicotinamide Adenine Dinucleotide
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NF-κB	Nuclear Factor-kappa B
NO	Nitric oxide
Nox	NADPH oxidase

n-Tyr	Nitrotyrosine
O_2^-	Superoxide Anion
OH·	Hydroxyl Radical
$ONOO^-$	Peroxynitrite
PLC	Phospholipase C
RAAS	Renin-angiotensin-aldosterone System
ROS	Reactive oxygen species
SAHA	Suberoyl Anilide Hydroxamic Acid
SDS	Sodium Dodecyl Sulfate
SHR	Spontaneously Hypertensive Rats
Sirt-1	Sirtuin 1
SNP	Sodium Nitroprusside
SODs	Superoxide Dismutases
TPR	Total Peripheral Resistance
VPA	Valproic Acid
VSMC	Vascular Smooth Muscle Cells
WKY	Wistar Kyoto
WHO	World Health Organization

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CHAPTER 1

Background and Literature Review

1. Hypertension

Hypertension is one of the most common, multi-factorial chronic disorder affecting over 1 billion people worldwide (Bloch 2016; Frid et al. 2020; Schwartz et al. 2012; Fields et al. 2004). According to the World Health Organization (WHO), sustained raised blood pressure (BP) equal to or above 140 mm Hg (systolic BP) and/or equal to or above 90 mm Hg (diastolic BP) is called hypertension. High BP is associated with increased risks of coronary and cerebrovascular events (Kjeldsen 2018; Petrie, Guzik, and Touyz 2018). Whilst there has been a gradual improvement towards treatment of hypertension, a great number of patients with increased BP remains resistant with the currently available treatment (Chia, Pandey, and Vongpatanasin 2019). Majority of the patients need more than one group of drugs to control the BP suggesting the involvement of multiple pathways in BP regulation (Guerrero-García and Rubio-Guerra 2018). However, the underlying mechanisms contributing to hypertension are very complicated and still remain obscure. Therefore, better understanding of the molecular mechanisms of hypertension will open the pathways to explore the new therapeutic strategies and reduce the prevalence of this global burden.

1.1. Types of Hypertension

Almost 90-95% hypertensive patients that do not exhibit any clear etiology are classified as having primary or essential hypertension whereas, rest of the 5-10% of patients are grouped under secondary or non-essential hypertension with an underlying and potentially reversible cause (Gupta-Malhotra et al. 2015).

In essential hypertension, BP rises with undetermined cause that increases risk for cerebral, cardiac, and renal events (Staessen et al. 2003). Evidence suggest that essential hypertension magnifies cardiovascular risk factors and target end-organ damage such as left-ventricular hypertrophy and cognitive dysfunction which finally lead to the catastrophic events such as stroke, heart attack and renal failure (Bolívar 2013). This is mostly common in adulthood and elderly person.

The common causes of non-essential hypertension are renal parenchymal disease, coarctation of the aorta, hyperaldosteronism, Cushing syndrome, thyroid disease etc. The prevalence of

secondary hypertension is more common in younger person and may vary by age from 18 to 40 years (Charles, Triscott, and Dobbs 2017).

1.2. Genetic analysis of Hypertension

Hypertension is a heterogeneous disease. Environmental factor as well as genetic predisposition both have strong influence on the rise of BP. The influence of genetics to the BP regulation is of two types (Agarwal, Williams, and Fisher 2005). One is the rare familial forms of monogenic hypertension that is considered as secondary hypertension as it is caused by a single gene and can be identified by a few hundred genetic markers. However, for the primary or essential hypertension, genotyping of a large number of small-effect size genetic variants are involved (Russo et al. 2018). Through Genome-wide association studies (GWAS) 43 of loci have been identified to be associated with systolic, diastolic BP, and hypertension (Newton-Cheh et al. 2009; Azam and Azizan 2018). It is therefore, opening a perspective on the genetic architecture of BP and facilitating the better understanding of genes that are implicated in the regulation of BP (Ehret and Caulfield 2013; Patel, Masi, and Taddei 2017).

2. Blood Pressure

Blood pressure (BP) is the force exerted on the arterial system of the body by the circulating blood pumped by the heart (Magder 2018). BP values are measured in millimeters of mercury (mmHg) and major determinants of therapeutic decisions. It is conventionally separated into systolic and diastolic determinations. **Systolic BP** is the maximum BP during contraction of the ventricles and pumping oxygen-rich blood into the blood vessels. Normal range for systolic BP is 100-140 mmHg with an average of 120 mmHg. **Diastolic BP** is the minimum pressure on the blood vessels when the heart muscle relaxes. The diastolic pressure is always lower than the systolic pressure. Normal range for diastolic BP is 60-90 mmHg with an average of 80 mmHg. The difference between systolic and diastolic pressure is called pulse pressure that represents the force exerted by the heart during each contraction. Another important variable is mean arterial pressure (MAP), that is the average arterial pressure throughout one cardiac cycle, systole and diastole and is considered as a better indicator of perfusion pressure to supply oxygenated blood to the vital organs of the body (Guyton et al. 1981).

2.1. Blood Pressure Regulation

Regulation of the circulatory system is critical to maintain a constant arterial pressure in ensuring adequate perfusion. BP depends on cardiac output (CO) and total peripheral resistance (TPR). CO is the volume of blood that is pumped out of the left ventricle per minute. TPR is the sum of all the blood vessels in systemic circulation. Changing any one of these factors will change the BP regulation. BP regulation is a complex physiological process operating in short-term and long-term reflex responses mediated by hormones, local vascular factors, and neural mechanism (Guyton et al. 1981).

2.2. Vascular Structure

Vascular Structure is playing an indispensable role in maintaining BP homeostasis by providing adequate blood supply to the tissues, and then returns it to the heart. The vascular system of the human body is comprised of many vessels. Blood primarily moves through the body by the rhythmic movement of smooth muscles in the vessel wall and by the action of the skeletal muscle as the body moves. There are three major types of blood vessels: the arteries, the capillaries and the veins (Pugsley and Tabrizchi 2000). **[Figure 1].**

Arteries carry blood away from the heart. The wall of an artery consists of three layers (Burton 1954). The innermost layer, the tunica intima that is made with a layer of endothelial cells (EC) and connective tissue. The middle layer, the tunica media, that mainly consists of vascular smooth muscle cells (VSMC) and connective tissues full of collagen, elastin, and other elastic fibers (Rhodin 1967). VSMC control the caliber of the vessel and arterial tone. The outermost layer, tunica externa that is made up of fibrous tissue, which is mostly elastin and collagen fibers, and fibroblasts. BP homeostasis is highly influenced by the elasticity of arteries. The largest artery, aorta contains large amounts of elastin and persistent with the pressure fluctuations from the heart. Whereas, small arteries have the large proportion of tunica media making them more muscular and active in vasoconstriction, allowing changes in TPR in response to the BP regulation. The small arteries, lumen (diameters <400 μm) are called resistance arteries because they act as the major site of vascular resistance. Any change in lumen diameter of these resistance vessels has influence in normal BP (Intengan et al. 1999).

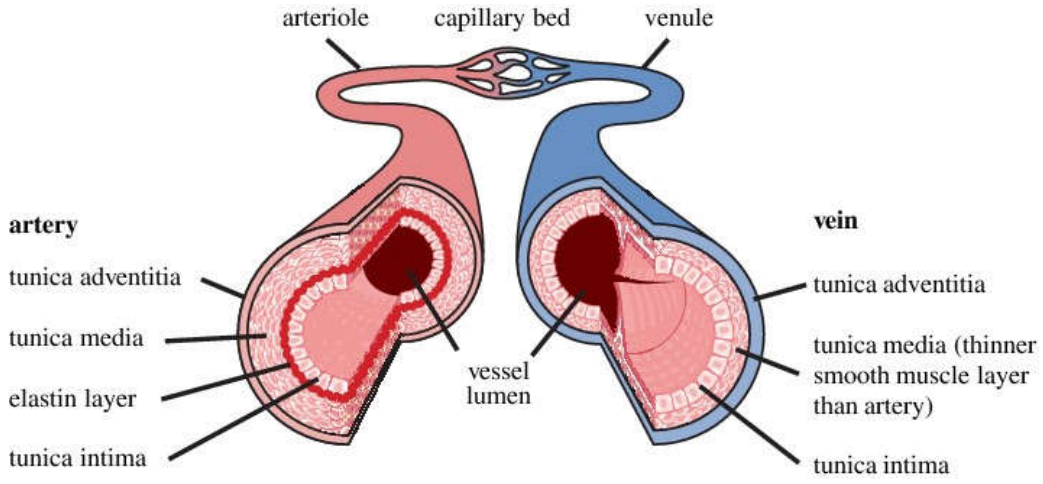

Figure 1: The Structure of the Arterial and Venous Vascular Wall. Adapted from (Shaw et al. 2014)

Capillaries are the smallest and most numerous of the blood vessels that mediate the connection between arteries and veins. Smooth muscle cells in the arteries help to regulate blood flow from the arterioles into the capillaries (Taylor, Moore, and Khimenko 1994).

Veins carry blood toward the heart. The walls of veins have the same three layers like the arteries but there is less smooth muscle and connective tissues. This makes the walls of veins thinner than those of arteries, because blood in the veins has less pressure than in the arteries (Klabunde 2011).

3. Vascular Remodeling

Vascular wall is an active organ composed of different types of cells including endothelial, VSMC and fibroblast cells that interact with each other to form an autocrine-paracrine complex. The vasculature undergoes structural and functional changes in response to long-term physiological alterations applied to the vessel walls such as increased transmural pressure and blood flow. These structural and functional alterations result in vascular remodeling (Gibbons and Dzau 1994). Vascular remodeling involves changes in at least four cellular processes: cell growth, cell death, cell migration, and the synthesis or degradation of extracellular matrix. Vascular remodeling is influenced by dynamic interactions between local growth factors, vasoactive substances, and hemodynamic stimuli (Intengan et al. 1999). Vascular remodeling is the hallmark

of the pathophysiology of vascular diseases and circulatory disorders including hypertension (Baumbach and Heistad 1989).

3.1. Vascular Remodeling in Hypertension

The histopathological change of hypertension is associated with vascular remodeling (Renna, de Las Heras, and Miatello 2013). The main characteristics of vascular remodeling are the thickening of the arterial wall including tunica intima, media, and externa. The thickening of arterial wall increases stiffness to reduce lumen diameter at a given pressure (Bund and Lee 2003). One of the major findings is that vascular tunica media thickening is caused by the abnormal proliferation and hypertrophy of VSMC (Inokuchi et al. 2001). More advanced findings indicated that several intracellular signaling pathways that regulate the expression of upstream and downstream target genes through cascade, are involved in the proliferation and hypertrophy of VSMC. Vasoactive peptides such as Ang II and ET-1 as well as growth factors receptors such as EGFR and PDGFR all contribute to VSMC hypertrophy and proliferation (Almajdoob, Hossain, and Anand-Srivastava 2018; Atef and Anand-Srivastava 2016; Inagami and Eguchi 2000; Gomez and Anand-Srivastava 2010; Li, Lévesque, and Anand-Srivastava 2010; Raines 2004; Ross et al. 1974). Therefore, understanding of cellular mechanisms involved in the proliferation, migration, and apoptosis of VSMC and the associated drug interventions may be a promising direction for the treatment of hypertension (Brown et al. 2018).

4. Spontaneously Hypertensive Rats

Spontaneously hypertensive rat (SHR) is the most common model of human hypertension that is genetically inherited hypertension. It has been widely used to define hypertension-induced changes in signaling mechanisms (Takata and Kato 1995) and to test new antihypertensive medication (Lerman et al. 2019; Doris 2017; Lund-Johansen 1990). This inbred strain was developed by Okamoto and colleagues during the 1960s, with the selective breeding of Wistar Kyoto (WKY) rats having hypertension (Okamoto and Aoki 1963). In SHR, development of hypertension begins at 4-week-old and increases with age (Li and Anand-Srivastava 2002). In adult (8-week- old) age, systolic BP reaches to around 180 mmHg (McGuire and Twietmeyer

1985). At the age of 40-weeks, SHR started to develop characteristics of cardiovascular disease, such as vascular and cardiac hypertrophy (Conrad et al. 1995). SHR demonstrates specific and uniform genetic predisposition that allow hypertension research including its causes, mechanisms and pathology, as well as possible therapeutic interventions (Folkow 1982). Like humans, male SHR shows to develop hypertension more rapidly and becomes more severe than female SHR (Iams and Wexler 1979). Moreover, increased media-to-lumen ratio observed in arteries from human hypertensive patients are identical to that observed in SHR (Heagerty et al. 1993). Thus, SHR provides a convenient approach of investigating hypertensive symptoms that are predictable and controllable without using life-threatening interventions for humans.

5. Consequences of Hypertension

Hypertension remains the leading cause of morbidity and mortality worldwide. High BP increases the risk of almost all the major cardiovascular events including stroke, sudden cardiac death, coronary heart disease, myocardial and cerebral infarction, abdominal aortic aneurysm, and peripheral vascular diseases (Kjeldsen 2018). Hypertension is also common among patients with diabetes mellitus (de Boer et al. 2017). The combined impacts of hypertension and diabetes can increase the risk of cardiovascular death. However, Oh et al. reported that hypertension is more strongly associated with all-cause and cardiovascular mortality than diabetes (Oh, Allison, and Barrett-Connor 2017). Hypertension is very frequent in patients with renal disease and its prevalence increases as renal failure progresses (Luo, Hu, and Jiang 2020). A considerable number of articles are devoted to pathophysiological and clinical aspects of hypertension-induced neurodegenerative and cognitive diseases. Hypertension leads to dementia and Alzheimer's disease that is a consequence of the damaging effects of high blood pressure on the cerebral vasculature (Carnevale et al. 2016; Gorelick et al. 2011).

6. Treatment of Hypertension

Hypertension associated risks of morbidity and mortality can be greatly reduced by treatment with antihypertensive drugs that lower BP. A total of 69 antihypertensive drugs are approved by the US Food and Drug Administration (FDA) (Oparil and Schmieder 2015). Initially, groups of antihypertensive drugs to promote vascular health included ACE inhibitors, calcium-channel

blockers, α-adrenergic blockers and thiazide-type diuretics (Officers 2002). Some newer cardiovascular drugs, such as antagonists of the Ang II receptor (AT 1), vasopeptidase inhibitors, dual acting ARB–neprilysin inhibitors, and Endothelin-1 (ET1) receptor blockers are considered as second-line drugs with the potentiality of improving vascular functions (Prasad, Palaniswamy, and Frishman 2009; Correale et al. 2018). Despite the plethora of available drugs, optimal treatment remains a challenge. One of the important facts is that many of these drugs do not specifically target the vascular system to ameliorate hypertension-induced vascular damage. Moreover, most of the cases, patients are prescribed to take medications from more than one drug groups. Therefore, it has been challenging to follow-up patients to prevent potential adverse drug interactions with a multidrug regimen (Grossman and Messerli 2012). Besides that, 10% to 15% patients with hypertension are still resistant with this currently available treatments (Persell 2011; Sim et al. 2013). However, current evidence suggests that development of treatment-resistant hypertension is multifactorial (Hwang et al. 2017). Thus, advanced knowledge to the understanding of vascular mechanisms responsible for high BP will facilitate efficient drug discovery to reduce the enormous clinical and economic burden for this population.

7. Molecular Mechanisms Implicated in Hypertension

Hypertension is a multifactorial disease. Several factors including vasoactive peptides, the renin-angiotensin-aldosterone system (RAAS), activation of the sympathetic nervous system, abnormalities in G protein-coupled receptor (GPCR) signaling, oxidative and nitrosative stress and inflammation are implicated in the pathophysiology of hypertension **[Figure 2]**. However, the underlying mechanisms for development of hypertension are very complicated and remain still poorly understood.

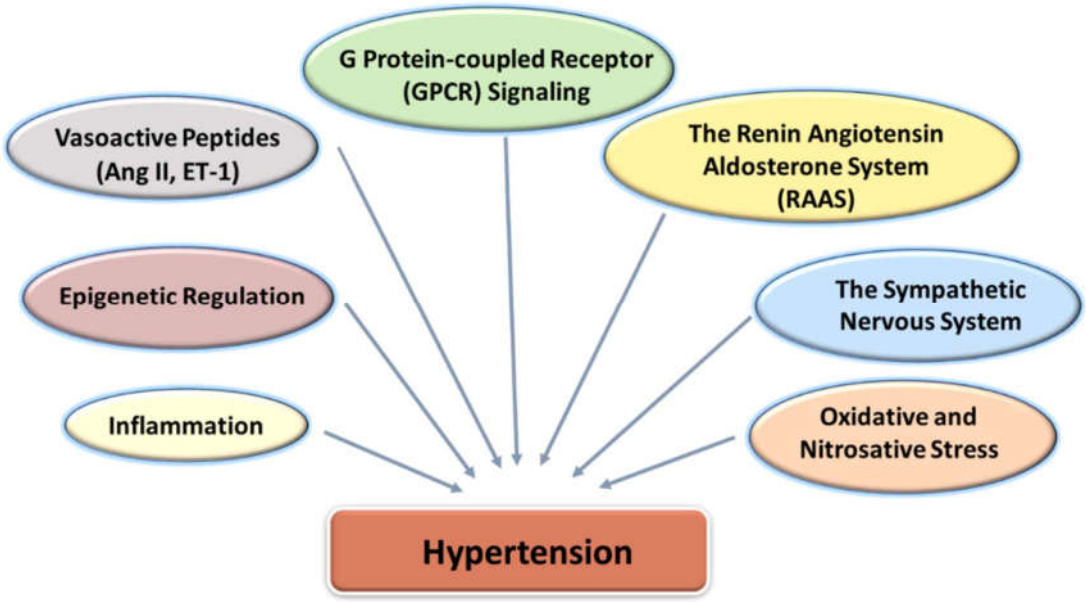

Figure 2: Several Factors Implicated in Hypertension Mechanism.

7.1. Role of Vasoactive Peptides in Hypertension

Vasoactive peptides including angiotensin II (Ang II), endothelin 1 (ET-1), vasopressin (AVP) and natriuretic peptides are upregulated in hypertensive patients and animal models (Hynynen and Khalil 2006; Arendse et al. 2019). The regulation of the vasoactive peptides results in endothelial dysfunction, vascular remodeling and vascular inflammation, which are implicated in the hypertension-induced vascular damage. Ang II, ET-1 and natriuretic peptides mediate their physiologic effects via G-protein-coupled receptors (GPCR) mediated signal transmission.

7.1.1. G-Protein Coupled Receptors (GPCR)

G-protein-coupled receptors (GPCR) are the most diverse family of membrane protein receptors. GPCR mediate most of our physiological responses to hormones, neurotransmitters and environmental stimulants, and possessed a great potential as therapeutic targets for a wide range of diseases (Rosenbaum, Rasmussen, and Kobilka 2009; Heng, Aubel, and Fussenegger 2013). Some examples of GPCR include beta-adrenergic receptors, which bind epinephrine; prostaglandin E2 receptors, which bind prostaglandin; angiotensin type I (AT1) receptor which binds Ang II (Fuxe et al. 2008). All GPCR are characterized by the presence of seven membrane-spanning α-helical segments interconnected by three extracellular loops (EL1, EL2, EL3)

containing the ligand binding domain and three alternating intracellular loops (IL1, IL2, IL3) provide binding sites for intracellular signaling proteins (Lomize, Pogozheva, and Mosberg 1999) **[Figure 3].** GPCR bind with extracellular stimuli and transmit signals to the effectors adenylyl cyclase (AC) or phospholipase C (PLC) through the activation of guanine nucleotide-binding proteins (G protein) (Wess 1997) that eventually generates numerous cellular events, such as increased heart rate in response to epinephrine (Gether 2000). The adenylyl cyclase-cyclic adenosine monophosphate (cAMP) signal pathway and PLC-phosphatidylinositol signal pathway are the two principal signal transduction pathways of the GPCR

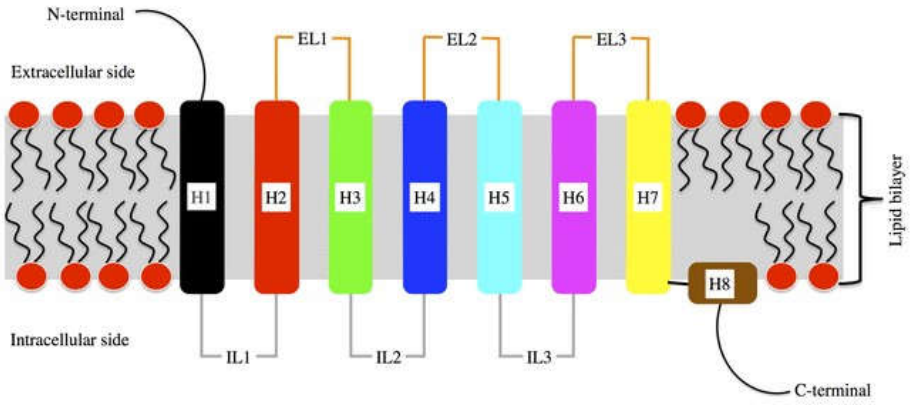

Figure 3: Schematic View of The Structure of G Protein-Coupled Receptor (GPCR), with depiction of the connectivity of the intracellular (IL) and extracellular (EL) loops between helices (H). Adapted from (Fosséprè et al. 2014).

7.1.2. Renin-Angiotensin System

The renin-angiotensin system (RAS) is the major regulatory system of blood pressure. The RAS system is composed of different regulatory components and effector peptides that mediate the dynamic control of vascular function (Nakagawa et al. 2020; Carey and Padia 2018). Renin is a protease, released from juxtaglomerular cells of the kidney and is the starting point of the renin-angiotensin system (Kukida et al. 2020; Hackenthal et al. 1990). Renin causes the cleavage of angiotensinogen, a substrate that is synthesized by the liver, into angiotensin (Ang) I. Ang I is proteolytically cleaved by the dipeptide carboxypeptidase, angiotensin-converting enzyme (ACE) and produces angiotensin II (Ang II). Ang II is further cleaved by the carboxypeptidase, ACE2 and produces Ang (1–7) peptide (Donoghue et al. 2000). Ang (1-7) binds with the GPCR *mas* receptor

and is involved in cardiovascular and neuronal regulation (Santos et al. 2003). Besides that, other Ang peptides, such as Ang III [Ang-(2-8)], Ang IV [Ang-(3-8)] are also produced from Ang II and may also have important biological activities (Hussain and Awan 2018).

Ang II is the dominant player of RAAS system. Ang II increases BP by vasoconstriction, sympathetic nervous stimulation, increased aldosterone biosynthesis and renal actions (Forrester et al. 2018). Ang II also contributes to high BP through the induction of growth and migration of VSMC, leading to thickening of the vascular wall and myocardium, and fibrosis (Touyz et al. 2018; Fyhrquist, Metsärinne, and Tikkanen 1995). Ang II mediates its effects through the activation of the two classes of GPCR: Ang II type 1 (AT1) receptor and Ang II type 2 (AT2) receptor (De Gasparo et al. 2000).

7.1.2.1. Angiotensin II AT1 Receptor

Most of the physiological actions of Ang II are mediated through AT1 receptor, which is widely expressed in the most cell types. The mechanism involves conformational changes of the receptor and coupling with the G protein, mainly Gqα and Giα (Shenoy and Lefkowitz 2005). Then, Gqα activates PLC and mediates downstream signaling (Lassègue et al. 1993; Kawai et al. 2017) . Giα is coupling to AC inhibition (Anand-Srivastava 1993) and activating of voltage-gated L-type and T-type calcium channels (Maturana et al. 1999). Ang II through the activation of AT_1 receptor promotes growth and stimulates extracellular matrix production in cardiac fibroblasts, cardiomyocytes, and VSMC. Clear evidence suggests that an excessive activation of the AT1 receptor results in hypertension and vascular remodeling (Billet et al. 2008) and central application of an AT1 receptor blocker or AT1 receptor antisense approach lowered BP in rodent hypertensive models (Yang et al. 1992; Toney and Porter 1993; Gyurko, Wielbo, and Phillips 1993). Ang II has also been shown to increase the expression of Giα proteins and hyperproliferation of VSMC (Gomez Sandoval, Levesque, and Anand-Srivastava 2009). In addition, our laboratory has also demonstrated the role of the endogenous Ang II and overexpression of Giα proteins in high BP in SHR because captopril, an ACE inhibitor that decreases the levels of Ang II also resulted in the attenuation of high BP and enhanced expression of Giα proteins (Pandey and Anand-Srivastava 1996).

7.1.2.2. Angiotensin II AT2 Receptor

AT2 receptor is highly expressed in the neonatal tissues, but rarely expressed in the cardiovascular system of the normal adults (Shanmugam, Corvol, and Gasc 1996). The role of AT2 on vascular responses to Ang II in humans remains controversial. An increased expression of AT2 receptor has been reported under pathological conditions including hypertension (Cosentino et al. 2005; Savoia et al. 2006), myocardial infarction (Nio et al. 1995) and vascular injury (Nakajima et al. 1995). Whereas, a protective role of AT2 receptors has been observed by inducing vasodilation, antiproliferation, and apoptosis in cellular and animal models (Volpe et al. 2003).

7.1.3. Endothelin System

Endothelin (ET-1) is a powerful vasoconstrictor peptide (21-amino-acids) that is diversely expressed and has important role in the vascular system. Three distinct isoforms of endothelin family have been identified; ET-1, ET-2, and ET-3. Structurally ET-2 and ET-3 differ from ET-1 by two and six amino-acid positions, respectively. All three endothelin isoforms are synthesized by two proteases as preprohormones and then post-translationally processed to active peptides. Firstly, the ~200-residue preproendothelins are proteolytically cleaved by endopeptidases to big ETs (37- to 41 amino acids called proETs). proETs is then cleaved by the endothelin converting enzyme (ECE) to the 21-amino acid mature active peptides (Inoue et al. 1989). However, ET-1 is most abundantly expressed. ET-1 is produced by VSMC, fibroblasts, cardiomyocytes, various brain neurons, but the predominant source of ET-1 is EC (Kisanuki et al. 2010; Kanse et al. 1991; MacCumber et al. 1989; Sakai et al. 1996; Yanagisawa et al. 1988). ET-2 is produced by intestinal epithelial cells, and ET-3 by neurons, renal tubular epithelial cells, and intestinal epithelial cells (Kedzierski and Yanagisawa 2001; Matsumoto et al. 1989). A wealth of evidence suggests that ET-1 is a key mediator in CVDs such as chronic heart failure and participates in the pathogenesis of the elevation of BP in both experimental animal models and human essential hypertension (Hynynen and Khalil 2006; Schiffrin 2001; Dhaun et al. 2008). Several animal models of hypertension such as DOCA-salt, Goldblatt (1K1C) and the SHR, showed an elevated systemic level of ET-1 (Kassab et al. 1997; Schiffrin 1995).

7.1.3.1. Endothelin-1 Receptors

There are two primary human endothelin receptors known; endothelin type A (ET_A) and type B (ET_B). They are the members of the seven transmembrane GPCR superfamily. Both receptors activate G proteins, leading to diverse responses such as activation of phospholipase C and increase in intracellular calcium (Inoue et al. 1989). ET_A is located abundantly on VSMC whereas, ET_B is highly expressed in EC, but also present in VSMC (Sakurai et al. 1990; Arai et al. 1990). However, the expression level of ET_B is increased in VSMC under vascular pathologic conditions (Batra et al. 1993).

Interaction of ET-1 with ET_A and ET_B receptors on VSMC activates phospholipase C-inositol triphosphate pathway and increases intracellular calcium level that causes VSMC contraction with the phosphorylation of myosin kinase (Miyauchi and Masaki 1999; Lüscher and Barton 2000). On the other hand, ET-1 interacts with ET_B receptors on EC and activates endothelial nitric oxide synthase (eNOS) which increases the release of nitric oxide (NO) and causes vasodilation (Lüscher and Barton 2000). Thus, activation of ET-1 leads to dual vasoregulatory effects. ET_A receptor inhibitors, BQ123 and BQ610 have been shown to lower BP in different animal models of hypertension including SHR suggesting the role of ET_A receptor in the pathogenesis of hypertension in SHR (Morand-Contant, Anand-Srivastava, and Couture 2010; Douglas et al. 1994; Cassinotti et al. 2018).

7.2. Role of Oxidative and Nitrosative Stress in Hypertension

Although mechanisms underlying hypertension are not yet fully elucidated, a considerable body of literature proposed that oxidative stress is one of the fundamental mechanisms responsible for the development of hypertension and other vascular diseases (Rodrigo, González, and Paoletto 2011; González et al. 2014; Baradaran, Nasri, and Rafieian-Kopaei 2014; Touyz 2004; Loperena and Harrison 2017).

Oxidative stress is an imbalanced state of systemic manifestation of reactive oxygen species (ROS), when the production of ROS exceeds antioxidant defense mechanism (Birben et al. 2012). ROS including superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH\cdot$) and peroxynitrite ($ONOO^-$) are generated within the vascular wall and play an active role in vascular

biology. ROS are generally produced at a low concentration and function as signaling molecules regulating vascular contractility and cell growth. However, an enhanced production of ROS that is not counterbalanced by the endogenous antioxidant mechanisms and decreased NO levels causing nitro-oxidative stress contributes to the pathology of diseases including hypertension (Li et al. 2014; Poli et al. 2004; Saha et al. 2011; Schieber and Chandel 2014; Touyz and Briones 2011; Yin et al. 2013). Several studies have reported an excessive amount of ROS in essential hypertensive patients and various animal models of hypertension (González et al. 2014; Rodrigo et al. 2007; Lappas, Daou, and Anand-Srivastava 2005; Gusan and Anand-Srivastava 2013; Rahali, Li, and Anand-Srivastava 2018; Saha, Li, and Anand-Srivastava 2008). ROS production is also enhanced in cultured VSMC and isolated arteries from hypertensive rats and humans (Lappas, Daou, and Anand-Srivastava 2005; Atef and Anand-Srivastava 2016; Touyz and Schiffrin 2001). Our laboratory has also demonstrated that VSMC from SHR exhibit enhanced oxidative stress due to the enhanced production of O_2^-, increased activity of NADPH oxidase and the overexpression of NADPH oxidase subunits, Nox2, Nox4, p47phox (Almajdoob, Hossain, and Anand-Srivastava 2018; Gusan and Anand-Srivastava 2013) and decreased levels of NO, eNOS and augmented levels of peroxinitrite ($ONOO^-$) (Hossain et al. 2018; Li et al. 2014).

7.2.1. Major Reactive Oxygen Species Molecules

Superoxide anion (O_2^-): Superoxide (O_2^-) is a highly reactive anion radical, which is produced by the one-electron reduction of molecular oxygen **[Figure 4]**. Superoxide can be generated from two major sources: the mitochondrial respiratory chain and nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase) enzyme (Murphy 2009; Jones 1994). Production of O_2^- is important because this acts as a progenitor for other ROS, including H_2O_2, $OH\cdot$ and $ONOO^-$. O_2^- can act as a reducing agent, donating its extra electron to form $ONOO^-$ with nitric oxide (NO), or as an oxidizing agent, to produce H_2O_2. Thus, increased production of O_2^- can be harmful in several ways including removing the beneficial effects of NO (Rubanyi and Vanhoutte 1986). In addition, several studies have demonstrated that O_2^- can act as a vasoconstrictor (Auch-Schwelk, Katusic, and Vanhoutte 1989; Cosentino, Sill, and Katusić 1994)

Hydrogen Peroxide (H_2O_2): Hydrogen peroxide (H_2O_2) is produced from O_2^- either by spontaneously or catalyzed by the enzyme superoxide dismutases (SOD) **[Figure 4]**. H_2O_2 is relatively more stable than O_2^-, as it doesn't contain any free radicals. H_2O_2 can readily diffuse across membranes and acts as a signaling molecule that is involved in vasodilatation, gene transcription, phosphatase activity, and activating other sources of ROS (Gough and Cotter 2011; Neill, Desikan, and Hancock 2002).

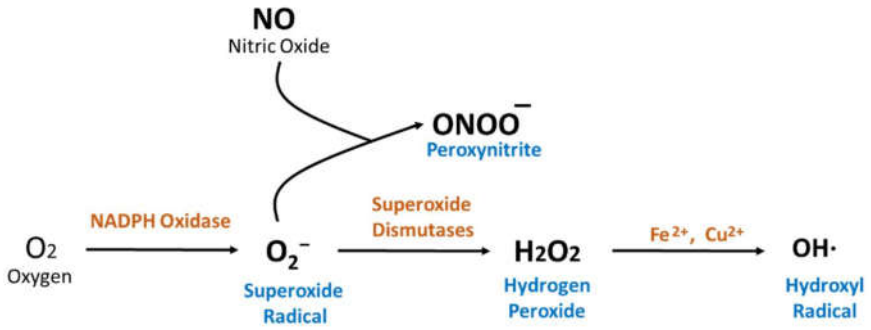

Figure 4: Sources and Formation of ROS in Mammalian Cells that are Associated with Hypertension

Hydroxyl Radical (OH·): Hydroxyl radical (OH·) is produced from the reaction between O_2^- and H_2O_2 where O_2^- donates 1 electron to H_2O_2 (Lipinski 2011) **[Figure 4]**. The hydroxyl radical is a highly reactive oxidant that can damage all types of macromolecules including carbohydrates, DNA, lipids (lipid peroxidation). The augmented level of OH· is reported to contribute to the increase contractions in the aorta of SHR (Auch-Schwelk, Katusic, and Vanhoutte 1989).

Peroxynitrite (ONOO–): Peroxynitrite ($ONOO^-$) is produced by the spontaneous reaction between O_2^- and NO **[Figure 4]**. $ONOO^-$ exerts exactly opposite of beneficial effects of NO. $ONOO^-$ is a very strong oxidant and can react with lipids, DNA, and proteins and causing oxidative damage to these macromolecules. Peroxynitrite can turn into novel products such as nitrotyrosine (n-Tyr), nitrotryptophan, and nitrated lipids that serve as important biological markers for many diseases. The ability of producing n-Tyr by peroxynitrite can impair signaling processes by inhibiting phosphorylation of critical tyrosine residues (Gow et al. 1996; Kong et al. 1996). Peroxynitrite generation contributes to various cardiovascular pathologies such as myocardial

and vascular dysfunction during ischemia and reperfusion, myocarditis, chronic heart failure and blood pressure regulation (Pacher, Beckman, and Liaudet 2007).

7.2.2. Source of ROS

ROS are produced in several cellular systems localized in the plasma membrane, the cytosol, peroxisomes, mitochondria and endoplasmic reticulum (Phaniendra, Jestadi, and Periyasamy 2015). The nicotinamide adenine dinucleotide phosphate (NADPH) oxidases are principal enzymatic sources of vascular ROS production in mammalian cells (Brandes and Kreuzer 2005).

7.2.2.1. NADPH Oxidase: Structure, Mechanism and Function

Vascular NADPH oxidases are by far the most researched topic amongst the sources of ROS in hypertension. NADPH oxidase is a membrane-bound, multi-subunit enzyme complex, that is distributed throughout the EC, VSMC and cardiac myocytes (Griendling et al. 2000; Xiao et al. 2002). It catalyzes the production of superoxide anion by transferring one electron to oxygen from NADPH **[Figure 5]**.

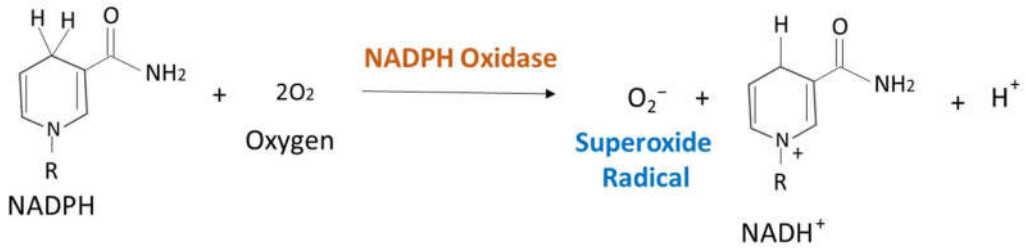

Figure 5: Reaction of the Production of Superoxide (O_2^-) by NADPH Oxidase

NAPDH Oxidase consists of two cytosolic subunits (p47phox and p67phox), a cytochrome b558 (gp91phox and p22phox) and a small G protein Rac. Gp91phox, also referred as Nox2 is an important Nox family member that is inactive until it binds to the membrane-anchored p22phox. The Nox2/p22phox complex requires phosphorylated p47phox, p67phox and p40phox for binding with the other cytosolic components. Upon complex assembly, the GTPase Rac then interacts with Nox2 and subsequently interacts with p67phox, resulting in an activated complex to produce superoxide through electron transfer from cytosolic NADPH to oxygen (Magnani and Mattevi 2019; Filip-Ciubotaru et al. 2016) **[Figure 6]**.

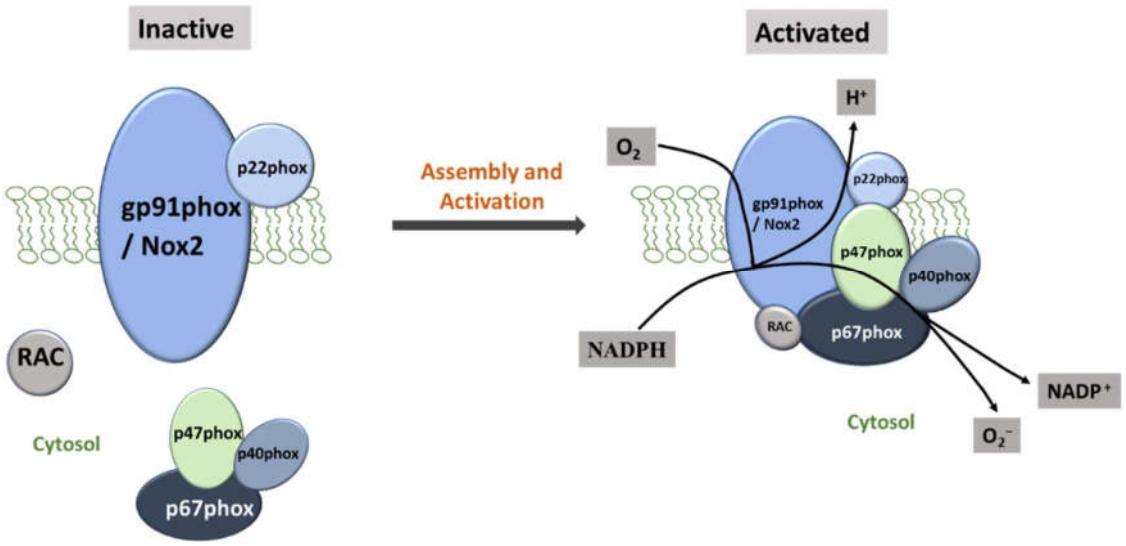

Figure 6: Assembly and Activation of NADPH Oxidase Subunits. The vascular NADPH oxidase also contains Nox1 and Nox4 as substitutes for gp91phox or Nox2. NADPH Oxidase comprises cytosolic (p47phox, p67 phox, p40 phox and Rac) and membrane subunits (gp91 phox and p22 phox). During activation, cytosolic subunits comprise a multi-component enzyme and migrate to the plasma membrane to dock with the membrane subunits. This multi-subunit enzyme produces a superoxide anion (O_2^-).

The subcellular distribution of Nox subunits varies according to cell type and localization. Human possesses six additional Nox2 homologues: Nox1, Nox3, Nox4, Nox5, DUox1, and DUox2 and all can participate in the catalytic reaction of the reduction of molecular oxygen to superoxide (Rada and Leto 2008). Nox1 contains structurally and functionally most similarity with Nox2 but is expressed in a low concentration in the VMSCs, fibroblast and EC (Li and Shah 2002). Nox4 is highly expressed in all vascular cells (Hilenski et al. 2004). Nox5 is expressed in human, but not in rats (Touyz et al. 2019). VSMC from SHR showed a markedly increased level of oxidative stress with the overproduction of superoxide anion (O_2^-) and enhanced activity of NAPDH oxidase as well as an over expression level of NAPDH oxidase subunits Nox1/Nox2/Nox4 and p47phox (Gusan and Anand-Srivastava 2013).

7.2.3. NO bioavailability and Hypertension

Accumulating evidence demonstrates that NO, produced by the endothelial nitric oxide synthase (eNOS) in the vascular endothelium, plays a critical role in regulating blood pressure (Hermann, Flammer, and Lüscher 2006; Demougeot et al. 2005). NO stimulates guanylyl cyclase to increase 3',5'-cyclic guanosine monophosphate (cGMP) production, which promotes vasodilatation on

VSMC (Archer et al. 1994; Tanaka et al. 2006), prevents from platelet adhesion and aggregation, exerts antiproliferative and antimigratory effects on EC and VSMC (Lüscher et al. 2001; Sandoo et al. 2010). Reduction in NO bioavailability is the hallmark of endothelial dysfunction and contributes to the development of hypertension and other vascular diseases (Brunner et al. 2005; Lüscher and Vanhoutte 1986; Panza et al. 1990). eNOS knockout animal models as well as Nω-nitro-l-arginine methyl ester (L-NAME)-induced inhibition of NO synthesis are reported to develop arterial hypertension (Huang et al. 1995; Arnal et al. 1993; Di Fusco and Anand-Srivastava 2000). Several mechanisms have been implicated in reduced NO bioavailability in hypertension (Li, Yon, and Cai 2015). Destruction of NO by superoxide anion is one of the major causes for reduced NO bioavailability. In the absence or reduced levels of cofactor, tetrahydrobiopterin (BH4) required to activate eNOS to produce NO, eNOS generates superoxide anion instead of NO and thereby results in enhanced oxidative stress. This is referred to as the eNOS uncoupling (Luo et al. 2014; Yang et al. 2009) and is one of the crucial mechanisms contributing to hypertension (d'Uscio 2011; Karbach et al. 2014). Therefore, increasing the NO signaling via restoration of eNOS coupling activity may serve as an important therapeutic strategy for hypertension (Li et al. 2006).

7.3. Transmembrane Signaling in Hypertension

7.3.1. Guanine Nucleotide-Binding Proteins

Heterotrimeric guanine nucleotide-binding proteins (G protein) are the largest family of signaling proteins. These proteins take part in the signal transduction through their interaction with the GPCR and thus, modulates the function of many downstream effectors (Wess 1997). G proteins are involved in myriad number of cellular processes and have become the efficacious therapeutic targets for diseases like cancer, CVD including hypertension. These proteins derived the name due to their ability to bind with the guanine nucleotides guanosine triphosphate (GTP) and guanosine diphosphate (GDP) and to have intrinsic GTPase activity (Watson et al. 1996). They mediate a molecular switch between two interchangeable states through the activation and termination of a variety of downstream signaling mechanisms including adenylyl cyclase/cyclic AMP (cAMP), Phospholipase C and calcium, MAP kinase pathway etc. Based on the sequence homology, all vertebrate G proteins belong to 4 major classes: Gs, Gi, Gq, and G12/13 (Downes

and Gautam 1999; Strathmann and Simon 1991). All G proteins are composed of three subunits: Gα, Gβ and Gγ (Neer and Clapham 1988). In the inactive state, Gα remains bound with Gβ/Gγ dimer and GDP molecule (Lambright et al. 1994) **[Figure 7].**

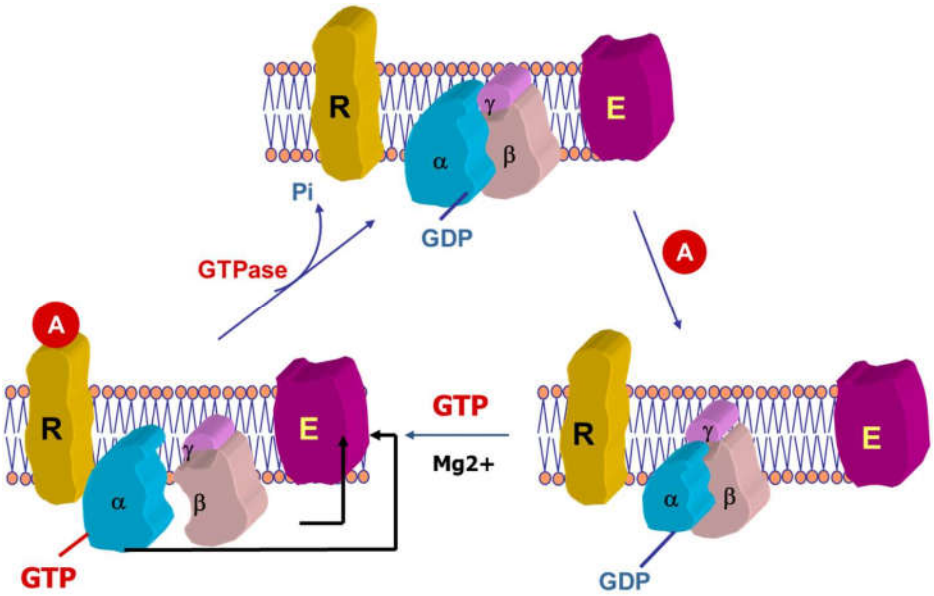

Figure 7: Activation of G-protein. Binding with a signaling molecule **(A)** to GPCR induces a conformation change that allows inactive G-protein to exchange of GDP for GTP on the α subunit of the heterotrimeric complex. Both GTP-bound Gα in the active form and the released Gβγ dimer can then go on to stimulate downstream effectors. After signaling mechanism, the GTP on Gα is hydrolyzed to GDP the original receptor is restored. (The diagram is adapted from a lecture by Dr Madhu B. Anand-Srivastava, PSL6090, 2015.)

When an agonist/signal molecule binds to GPCR, the GPCR undergoes a conformational change that activates the G proteins by promoting the exchange of Gα bound GDP to GTP. This leads to the dissociation of Gβ/Gγ dimer from Gα (Oldham and Hamm 2008). Now, activated Gα-GTP acts upon their downstream effectors and thereby initiate intracellular signaling responses. However, after the signaling mechanism, GTP from Gα-GTP is hydrolyzed to GDP and produces inactive state of G protein where Gα bound GDP (Gα-GDP) re-associates with Gβ/Gγ dimer to form the heterotrimeric structure (Tuteja 2009) **[Figure 7].**

7.3.2. Adenylyl Cyclase/cAMP Signaling

Adenylyl Cyclase/cAMP signal transduction system has been targeted for the treatment of CVDs from decades (Gold, Gonen, and Scott 2013). When a hormone binds to GPCR, activated Gα is released to transmit signal on the effector molecule called adenylyl cyclase (AC). AC then converts

adenosine triphosphate (ATP) to cyclic adenosine monophosphate (cAMP) and increases cAMP level. cAMP is a second messenger which has major implications in numerous cellular functions by activating enzyme protein kinase A **[Figure 8]**.

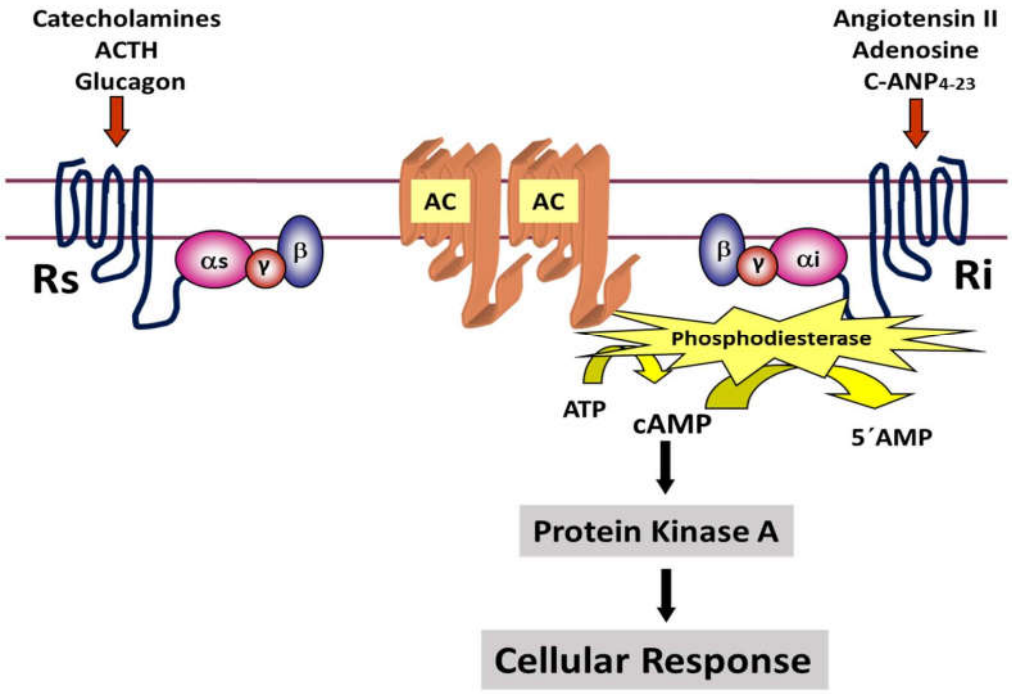

Figure 8: G-protein Mediated Adenylyl Cyclase/cAMP Signaling. Where, AC is denoting as adenylyl cyclase. Rs and Ri are the stimulatory GPCR and inhibitory GPCR, respectively. (The diagram is adapted from a lecture by Dr Madhu B. Anand-Srivastava, PSL6090, 2020.)

Aberrant activation of cAMP can have pathophysiological consequences related to cardiac contractility, vascular tone and reactivity etc. (Asano, Masuzawa, and Matsuda 1988; Baumann et al. 1981). Basal activity of AC is reduced in SHR resulting in reduced production of cAMP associated with the hyperproliferation of VSMC and endothelial dysfunction in SHR (Gusan and Anand-Srivastava 2013; Shah and Singh 2006). However, activating or inhibiting AC signal propagation is regulated by G proteins (Gilman 1989). G protein, responsible for stimulating or activating AC and increased cAMP production is called G-stimulatory (Gs) protein, whereas, inhibition of AC and reduced cAMP production is regulated by G-inhibitory (Gi) protein.

7.3.2.1. G-stimulatory (Gs) Protein

The alpha subunit of G-stimulatory protein (Gsα) is positively coupled to AC and mediates the stimulatory responses of hormones upon activating AC, thereby increases cAMP messenger level. Molecular cloning of human cDNA has revealed four distinct types of Gsα resulting from differential splicing of an individual gene (Bray et al. 1986; Robishaw, Smigel, and Gilman 1986). Gs protein has been shown to regulate intracellular calcium homeostasis and induces phosphorylation of contractile filaments. A reduced stimulation of Gs protein is associated with the β-adrenoceptor downregulation, that contributes to cardiac complications in SHR (Asano, Masuzawa, and Matsuda 1988).

7.3.2.2. G-inhibitory (Gi) Protein

The alpha subunit of G-inhibitory protein (Giα) is negatively coupled to AC and inhibits AC activity from cAMP production. Based on the sequence identity of their alpha subunit, these proteins are classified into subfamilies: Giα-1, Giα-2, Giα-3, GoαA, GoαB (Kehrl 1998). All three forms of Giα-1-3 take part in the AC inhibition and activation of atrial acetylcholine activated potassium channel. Dysfunction of Gi protein mediated signaling pathways has significant role in metabolic diseases like obesity and diabetes (Kimple et al. 2014). An absence of Giα-2 proteins has been shown to contribute in dilated cardiomyopathy and increased mortality in β1-adrenoceptor-overexpressing mice (Keller et al. 2015). An overexpression of Giα protein is thought to be one of the pathological factors contributing to hypertension and vascular remodeling in different hypertensive animal models (Ali El-Basyuni, Li, and Anand-Srivastava 2016; Anand-Srivastava, de Champlain, and Thibault 1993; Anand-Srivastava, Picard, and Thibault 1991; Ge, Garcia, and Anand-Srivastava 1999; Li and Anand-Srivastava 2002; Li et al. 2014; Sarkar, Li, and Anand-Srivastava 2019).

7.3.2.2.1. Role of Giα Protein Overexpression in the Pathogenesis of Hypertension

Gi proteins and associated signaling mechanisms are involved in maintaining vascular tone, contractility, proliferation and hypertrophy of VSMC (Almajdoob, Hossain, and Anand-Srivastava 2018; Atef and Anand-Srivastava 2014; Bou Daou, Li, and Anand-Srivastava 2016; Gomez

Sandoval et al. 2013; Rodbell et al. 1971). Thus, the impaired functions of Gi proteins results in developing hypertension and associated complications.

Our laboratory showed that Giα-2 and Giα-3 proteins and their mRNA but not of Goα and Gsα are overexpressed in heart and aorta from different hypertensive rat models including SHR, DOCA-salt, L-NAME-induced and 1 kidney-1clip hypertensive rats (Anand-Srivastava 1992; Anand-Srivastava, de Champlain, and Thibault 1993; Anand-Srivastava, Picard, and Thibault 1991; Di Fusco and Anand-Srivastava 2000; Ge, Garcia, and Anand-Srivastava 1999; Ge, Garcia, and Anand-Srivastava 2006; Lappas, Daou, and Anand-Srivastava 2005; Thibault and Anand-Srivastava 1992). Moreover, VSMC and lymphocytes from 12-week old SHR also showed an overexpression of Giα protein when compared to normotensive WKY rats (Lappas, Daou, and Anand-Srivastava 2005; Marcil and Anand-Srivastava 2001). Further studies confirmed that enhanced expression of Giα-2 and Giα-3 proteins in heart and aorta precedes the development of hypertension in SHR and DOCA-salt rats (Marcil, de Champlain, and Anand-Srivastava 1998; Marcil, Thibault, and Anand-Srivastava 1997). These studies suggest that enhanced expression of Giα proteins that results in decreased production of cAMP, could be one of the contributing factors in the pathogenesis of hypertension. This was further supported by the studies showing that inactivation or inhibition of Giα proteins by pertussis toxin or by antisense of Giα-2 protein attenuated the development of high BP in SHR (Ali El-Basyuni, Li, and Anand-Srivastava 2016; Li and Anand-Srivastava 2002; Triggle and Tabrizchi 1993). In addition, resveratrol, SNP, and C-ANP_{4-23}, a natriuretic peptide receptor C agonist have also been shown to attenuate the development of hypertension in SHR through the inhibition of overexpression of Giα proteins (Hossain et al. 2018; Li et al. 2014; Sarkar, Li, and Anand-Srivastava 2019).

The enhanced expression of Giα proteins in SHR was shown to be attributed to the augmented levels of endogenous Ang II, ET-1 and growth factors because the inhibition of AT1 receptor, ET_A receptor and growth factor receptors by pharmacological inhibitors or siRNA attenuated the overexpression of Giα proteins in VSMC from SHR (Sandoval Gomez, Li, and Anand-Srivastava 2011; Sandoval Gomez and Anand-Srivastava 2011). In addition, our laboratory by using the inhibitors or siRNA has also demonstrated that endogenous Ang II and ET-1 through the interaction with AT1 and ET_A receptor respectively increased the oxidative stress which through

the activation of c-Src and growth factor receptor augments the activity of MAP kinase and contributes to the enhanced expression of Giα proteins (Sandoval Gomez and Anand-Srivastava 2011; Sandoval Gomez, Li, and Anand-Srivastava 2011). Enhanced expression of Giα proteins results in the decreased levels of intracellular cAMP and increased vascular resistance and thereby leads to high BP **[Figure 9]**.

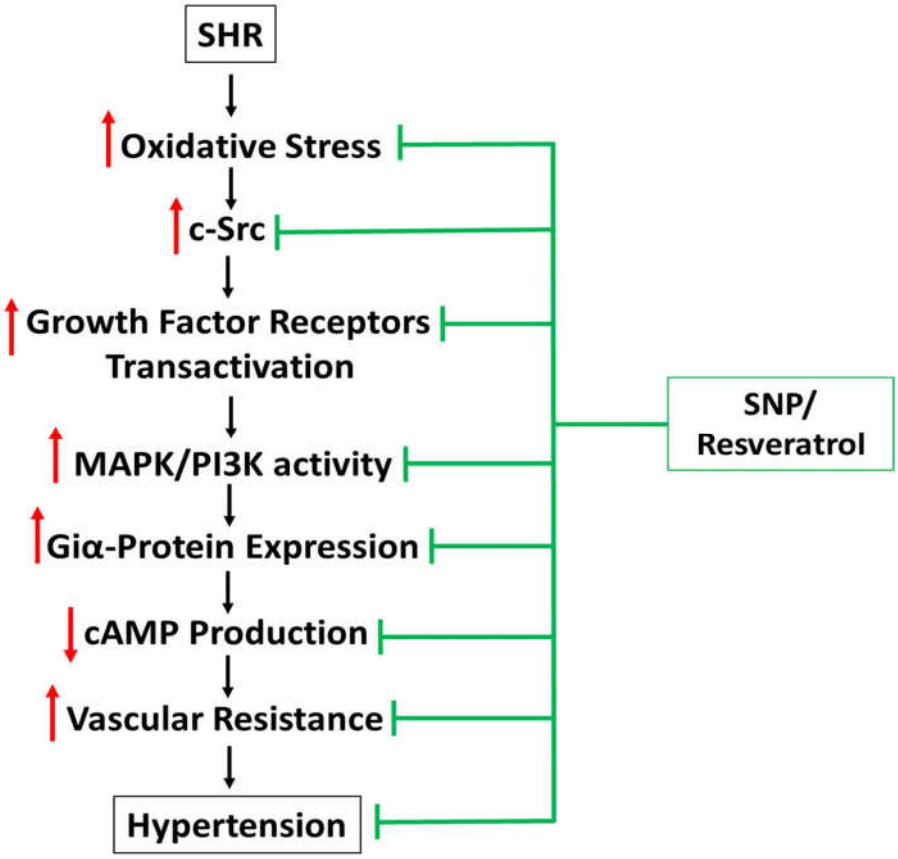

Figure 9: Schematic Diagram Summarizing the Effect of SNP/Resveratrol on Hypertension of SHR and the Implicated Molecular Mechanisms

In addition, SNP and resveratrol-induced attenuation of overexpression of Giα proteins and high BP in SHR was also shown to be attributed to the inhibition of oxidative stress and associated signaling mechanisms **[Figure 9]** (Hossain et al. 2018; Sarkar, Li, and Anand-Srivastava 2019). C-ANP_{4-23}-induced attenuation of high BP in SHR was associated with the inhibition of overexpression of Giα proteins and nitroxidative stress (Li et al. 2014).

7.3.2.2.2. Role of Giα Protein Overexpression in the Pathogenesis of Tachycardia

Giα protein signaling is a critical mediator of the regulation of heart rate modulation and dynamics (Ang, Opel, and Tinker 2012; Sebastian et al. 2013). A mutated GTP binding domain of Giα-2 protein has been shown to be responsible for the development of idiopathic ventricular tachycardia in human (Lerman et al. 1998). Nagata et al showed that Giα-2 (but not Giα-1/Giα-3) deficient mice resulted in inhibition of β-adrenergic receptor–induced contractility and calcium currents in adult murine cardiomyocytes (Nagata et al. 2000). However, SHR typically showed an increased heart rate and inhibition of the overexpression of Giα-2 attenuates BP and tachycardia (Ali El-Basyuni, Li, and Anand-Srivastava 2016; Li et al. 2014). Our laboratory has also demonstrated the role of Giα-2 but not of Giα-3 in tachycardia in SHR (Ali El-Basyuni, Li, and Anand-Srivastava 2016).

8. Histone deacetylase (HDAC)

Histone modifications includes acetylation or deacetylation of lysine residues, that play a key role in the epigenetic regulation of gene transcription (Berger 2002). Histone deacetylases (HDAC) are a group of enzymes that remove acetyl moieties from an ε-N-acetyl lysine amino acid on a histone and some other non-histone proteins (Ito, Barnes, and Adcock 2000; Glozak et al. 2005). Thus, HDAC controls chromatin remodeling (Felisbino et al. 2013) and alters expression of genes implicated in regulating several cellular processes (Felisbino et al. 2013). Based on sequence homology and phylogenetic analysis, four diverse classes of HDACs have been identified and are numbered from I to IV. Class I includes HDAC1, HDAC2, HDAC3, and HDAC8; Class II are HDAC4, HDAC5, HDAC6, HDAC7, HDAC9, and HDAC10; Class III are known as Sirtuin that include Sirt-1, Sirt-2, Sirt-3, Sirt-4, Sirt-5, Sirt-6, and Sirt-7; and class IV has a solely member HDAC11 **[Figure 10]**. Class I, II and IV HDAC are Zn^{2+}-dependent enzymes, which diverge significantly from the Class III HDAC that are dependent on nicotinamide adenine dinucleotide (NAD^{+}) for their catalytic activity (Holbert and Marmorstein 2005).

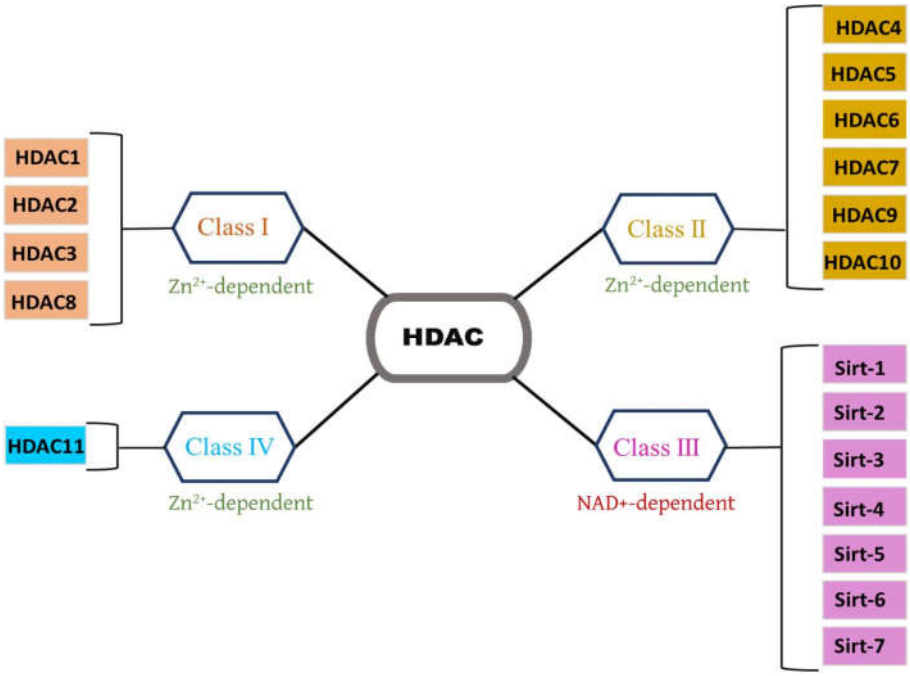

Figure 10. Histone Deacetylases (HDAC) Classifications. HDAC are divided into four classes, CLASS I (HDAC1, HDAC2, HDAC3, HDAC8); CLASS II (HDAC4, HDAC5, HDAC6, HDAC7, HDAC9, HDAC10); CLASS III (Sirt-1, Sirt-2, Sirt-3, Sirt-4, Sirt-5, Sirt-6, Sirt-7) and CLASS IV (HDAC11). Sirt- denotes as Sirtuin.

8.1. HDAC Inhibitors

A delicate balance between acetylation and deacetylation ensures an accurate gene regulatory event within the cell. Therefore, disturbance of HDAC activities may contribute to a varied range of diseases, from diabetes to cancer. For example, HDAC1 and HDAC2 show an overexpression in colon cancer (Yang et al. 2014), HDAC3 is upregulated in gastric cancers (Xu et al. 2018), HDAC7 exhibits an increased expression level in pancreatic islets from patient with type 2 diabetes (Daneshpajooh et al. 2017), HDAC6 has an higher expression in various neurodegenerative diseases (Simões-Pires et al. 2013). HDAC inhibitors exploit Zn^{2+}-binding sites and block the catalytic activity of HDAC. As a result, an increase acetylation of the histone promotes re-expression of silenced controlling genes, thus, emerging as potential therapeutic agents (Lombardi et al. 2011). Vorinostat, Romidepsin, Belinostat, and Panobinostat are the HDAC inhibitors which received FDA approval and are currently used as anti-tumor agents (Suraweera, O'Byrne, and Richard 2018).

8.2. HDAC in Vascular Remodeling, Emerging Therapeutic Targets for CVDs

Along with anti-tumor effect, HDAC inhibitors were recently shown to exhibit beneficial effects on vascular remodeling and cardiac function, thereby providing a novel cardiac therapeutic approach. Substantial studies showed that using various Zn^{2+}-dependent HDAC inhibitors is efficacious against several CVDs including cardiac arrhythmia, myocardial infarction, cardiac remodeling, hypertrophy, hypertension, and cardiac fibrosis (Yoon and Eom 2016). Increased expression of HDAC was shown to be implicated in increased proliferation, migration and hypertrophy of VSMC and associated diseases (Pietruczuk and Srivastava 2017). It was reported that overexpression of HDAC4 stimulated hyperproliferation and migration of VSMC and inhibition of HDAC4 effectively suppressed the proliferation (Zheng et al. 2019). Scriptaid, a broad spectrum HDAC inhibitor was also shown to attenuate mitogen-induced proliferation and neointimal hyperplasia in VSMC (Findeisen et al. 2011).

Inhibitory activity of class I HDAC has been shown to block Ang II-induced cardiac hypertrophy in mice or rats (Kee et al. 2006). Selective Inhibition of HDAC8 with PCI34051 also reduced the high BP through improving vascular remodeling in Ang II-induced hypertensive mice (Kee et al. 2019). Inhibition of Class I HDAC was also shown to attenuate hypertension and cardiac remodeling in SHRs (Cardinale et al. 2010). Taken together, it may be suggested that HDAC inhibitors are capable to reduce vascular remodeling, however, in depth-studies are needed to establish a potential therapy of CVDs.

9. Sirtuins, Class III HDAC

Sirtuins have garnered a remarkable attention in a short time since their discovery as key regulators of yeast replicative lifespan in 1955 (Kennedy et al. 1995). They are named after its founding member silent information regulator 2 gene (SIR2), that is vital for silencing heterochromatin in budding yeast, *Saccharomyces cerevisiae* (Rine and Herskowitz 1987). Mammalian sirtuins primarily catalyze the deacetylation of lysine residues on histones and various non histone proteins and some of them also possess ADP-ribosyl transferase activity (Liszt et al. 2005; Frye 1999). They are highly conserved, evolutionarily NAD+-dependent HDAC. There are seven known isoforms (Sirt-1–7) of sirtuins which participate in a wide range of cellular

processes and pathways with diversified cellular localization and molecular targets (Cen, Youn, and Sauve 2011). Each of them is comprised of a conserved 275 amino acid catalytic core and flanked by unique additional N-terminal and/or C-terminal region of variable length (Frye 2000). However, they vary in their sub-cellular localization: Sirt-1, Sirt-6 and Sirt-7 are located in the nucleus, Sirt-2 is prominent in the cytoplasm, and Sirt-3, Sirt-4 and Sirt-5 are found in mitochondria (Michishita et al. 2005; North et al. 2003) **(Table 1)**. Function wise, Sirt-1 and Sirt-5 show deacetylase activity (Vaziri et al. 2001; Kumar and Lombard 2018), Sirt-4 and Sirt-6 have ADP-ribosyl transferase activity (Haigis et al. 2006; Liszt et al. 2005), whereas Sirt-2 and Sirt-3 exhibit both activities (Shi et al. 2005; North et al. 2003). Sirt-7 has not been shown to possess NAD-dependent deacetylase nor an ADP-ribosyl transferase activity (North et al. 2003).

Table 1: Functional and Localized Diversity of Mammalian Sirtuin Protein Family.

Sirtuin	Functional Properties	Localization
Sirt-1	Deacetylase activity	Nucleus
Sirt-2	Deacetylase and ADP-ribosyl transferase activity	Cytoplasm
Sirt-3	Deacetylase and ADP-ribosyl transferase activity	Mitochondria
Sirt-4	ADP-ribosyl transferase activity	Mitochondria
Sirt-5	Deacetylase activity	Mitochondria
Sirt-6	ADP-ribosyl transferase activity	Nucleus
Sirt-7	?	Nucleus

9.1. Sirtuins in Cardiovascular System

All sirtuins are broadly implicated in cellular aging and longevity (Grabowska, Sikora, and Bielak-Zmijewska 2017; Satoh, Imai, and Guarente 2017; Camins et al. 2010; Bonkowski and Sinclair 2016). Besides that, they play certain roles in the regulation of energy metabolism, DNA repair,

maintenance of genomic stability, inflammation, neuroendocrine regulation and cardiac rhythm (D'Onofrio et al. 2015; Haigis and Guarente 2006; Michan and Sinclair 2007). The role of Sirtuin in vascular dysfunction and pathophysiology of cardiac diseases has been extensively studied in past years. Sirt-1 is the most critical regulator of the vascular functions. It is involved in modulation of angiogenic and vasodilatory function in EC and VSMC. Particular role and regulation by Sirt-1 in vascular homeostasis and diseases will be discussed in section 10. Deficiency of Sirt-2 has been shown to regulate microtubule function in EC by blocking Ang II-induced EC migration, which suggests a promising role of Sirt-2 in hypertension-induced vascular remodeling (Hashimoto-Komatsu et al. 2011). Activation of Sirt-3 regulates energy metabolism, protects cardiomyocytes by controlling systemic levels of oxidative stress and blocks the development of cardiac hypertrophy (Sun et al. 2018). Sirt-4 overexpression was reported to impair cardiac function and to exacerbate Ang II-induced cardiac hypertrophy suggesting a critical regulatory role of Sirt-4 in cardiac hypertrophy (Luo et al. 2016). Sirt-5 overexpression was shown to be protective for cardiomyocytes by inhibiting oxidative stress-induced apoptosis whereas, lack of Sirt-5 gene showed a significant reduction in cell viability (Liu et al. 2013). Sirt-6 is another functionally important sirtuin, like Sirt-1. Sirt-6 plays a critical role to prevent cardiac hypertrophy and heart failure, and suppresses tumor growth, participates in the regulation of VSMC differentiation and most remarkably prevents vascular endothelial dysfunction (Yepuri and Ramasamy 2019; Li et al. 2017; Cai et al. 2012; Yao et al. 2014; Sebastián et al. 2012). Recent study indicates a therapeutic potential of endothelial Sirt-6 in the prevention of hypertension and associated cardiorenal injury (Guo et al. 2019). Sirt-7 has been shown to participate in myocardial tissue repair mechanism as well as in repairing vascular lesions through the inhibition of VSMC proliferation (Araki et al. 2015; Kimura et al. 2017).

Thus, Sirtuins present an interesting therapeutic target in the prevention of cardiovascular diseases. It is assumed that clinical manipulation of sirtuins may be achievable without the costly and time-consuming process of discovering new drug targets. Therefore, small molecules, pharmacological modulators both activators and inhibitors, have been targeted and characterized for different sirtuins for the treatment and prevention of cardiovascular disease (Dai et al. 2018; Villalba and Alcaín 2012).

10. Sirtuin 1 (Sirt-1), Structure and Mechanism of Action

Sirtuin 1 (Sirt-1) has been the most widely studied member of NAD^+-dependent class III HDACs. It has been involved in aging, vascular homeostasis, energy balance, neuronal signaling, inflammatory responses, cell fate and stress responses, DNA damage responses and cancer. Sirt-1 deacetylates H1 (K26), H3 (K9 & K14) and H4 (K16) histone (Rifaï et al. 2018; Vaquero et al. 2004) and non-histone protein, including transcription factors (e.g., p53, NF-κB, FOXO, PGC-1α) and DNA repair proteins (e.g., Ku70, PARP1) (Giannakou and Partridge 2004; Han et al. 2017; Jeong et al. 2007; Liu, Liu, and Marshall 2009; Yeung et al. 2004). Mammalian Sirt-1 is a 747 amino acids (aa) sequence, with four different regions: N-terminal region (aa 1-182), allosteric site (aa 183-243), catalytic core (aa 244-498), and C-terminal region (aa 499-747). The N- and C-terminal regions are highly required for the sirtuin activity (Pan et al. 2012). More specifically, the catalytic activity depends on a 25 amino acid sequence (aa 631-655) at the C-terminal region (Davenport, Huber, and Hoelz 2014). N-terminal region is crucial to allow the nucleo-cytoplasmic translocation of Sirt-1 to facilitate its functional regulation (Tanno et al. 2007). The Sirt-1 catalytic reaction for protein deacetylation takes place in the catalytic core domain **[Figure 11]**.

Catalytic core consists of two sub-domains; one is specific for NAD^+ and another is for substrate binding (Davenport, Huber, and Hoelz 2014). When NAD^+ binds to the specific pocket at the subdomains' interface, then acetylated protein substrate binds to a small zinc domain next to the NAD+ binding site. NAD+ binding modifies catalytic core to increase substrate protein access (Imai et al. 2000). This structural arrangement revealed that Sirt-1 catalytic reaction is NAD+ dependent and proceeds by a sequential mechanism. NAD^+ and acetylated substrates both form a Sirtuin complex, removes the acetyl group from substrate protein, high-energy bond in NAD+ breaks down and a novel product, 2'-O-acetyl-ADP ribose is synthesized with nicotinamide (NAM) (Moazed 2001) **[Figure 11]**.. For the Sirt-1 inhibition, inhibitors block the NAD^+ binding sites or interfere with the acetylated protein binding, as a result, the catalytic activity of Sirt-1 becomes inactivated (Avalos, Bever, and Wolberger 2005) whereas activators of Sirt-1 bind to the allosteric site and positively regulate Sirt-1 catalytic activity (Cao et al. 2015)

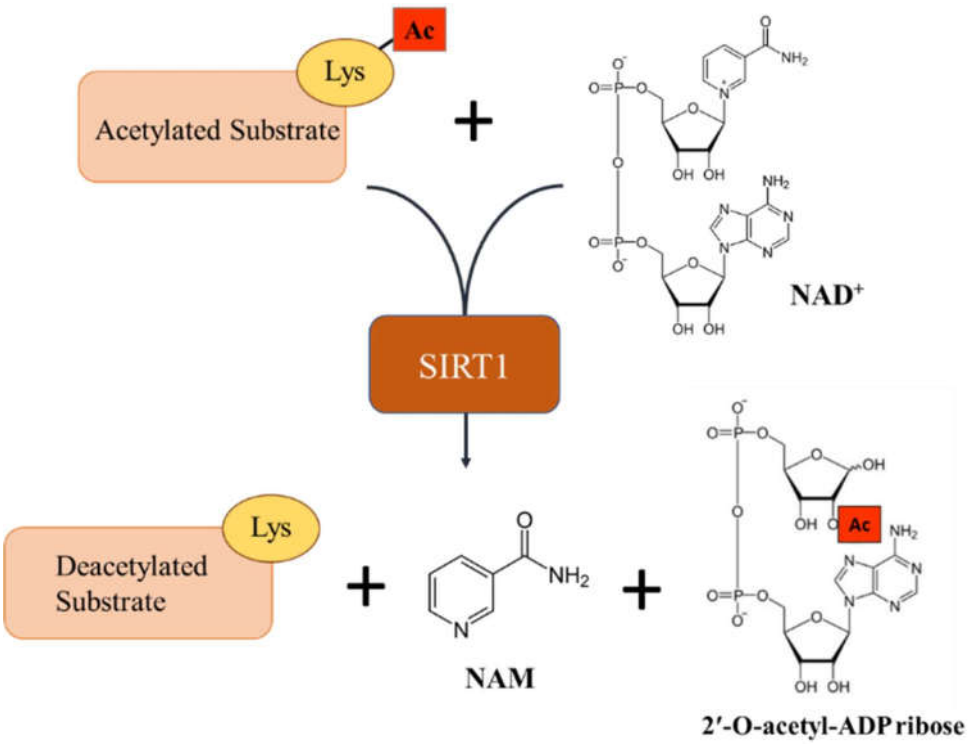

Figure 11: NAD$^+$-dependent Enzymatic Deacetylation Reaction of Sirt-1. Where Lys, Ac, NAM denote Lysine, Acetyl group and Nicotinamide respectively.

11.Sirt-1, as a Therapeutic Target

11.1. Sirt-1 in Nitroxidative Stress

Several studies suggest a strong connection of Sirt-1 with antioxidant mediated responses, that mediate signaling mechanism involved in the regulation of gene expression in cells exposed to oxidative stress. Resveratrol, a putative Sirt-1 activator has been shown to reduce oxidative stress via AMPK/Sirt-1-independent pathway in type 2 diabetic mice (Kitada et al. 2011). Resveratrol was also shown to prevent H_2O_2-induced damage by increasing Sirt-1 expression level in human endothelium (Kao et al. 2010). Small molecule Sirt-1 activator, SRT2104 showed to increase the antioxidant responses against age-dependent and ROS-mediated mitochondrial dysfunction in mice (Mercken et al. 2014). Though, there is a clear indication that Sirt-1 activation is protective against oxidative stress, multiple studies also suggest Sirt-1 overexpression as a contributing factor for the oxidative stress (Chen, Wan, and Liu 2013; Chong et al. 2012; Salminen, Kaarniranta, and Kauppinen 2013). Thus, relationship between Sirt-1 activity and ROS signaling

remains to be clarified and is crucial for the therapeutic use of Sirt-1. Studies have shown that Sirt-1 activation may have a protective effect against cardiac hypertrophy (Matsushima and Sadoshima 2015; Oka et al. 2011), however, in contrast, transgenic mice that have 12.5-fold increased expression of Sirt-1 were also reported to exhibit enhanced oxidative stress, an increased hypertrophy and decreased cardiac function (Alcendor et al. 2007) and indicates the dose dependent effect of Sirt-1 on oxidative stress.

Substantial literatures demonstrate that Sirt-1 deacetylation regulates transcription factors, including FoxO factors, nuclear factor-kappa B (NF-κB) and NRF2 that are involved in the regulation of cellular nitroxidative balance. Overexpression of Sirt-1 decreases the acetylation of FoxO factors, (FoxO1, FoxO3a and FoxO4) and helps to stimulate the expression of antioxidant gene, e.g., manganese superoxide dismutase (MnSOD), catalase via an auto-feedback loop which also suggests that Sirt-1 is a co-activator for FoxO (Daitoku, Sakamaki, and Fukamizu 2011). Sirt-1 inhibits NF-κB signaling, a major inducer of inflammatory responses; through deacetylation of the p65 subunit of NF-κB, to reduce inflammatory responses and ROS production (Rajendran et al. 2011; Salminen et al. 2008; Yang et al. 2012). NF-κB activates the expression of NADPH oxidase components, e.g., gp91phox and p22phox, which leads to increase in the free radical production (Anrather, Racchumi, and Iadecola 2006; Manea et al. 2007; Manea et al. 2015). NF-κB signaling also transactivates the inducible nitric oxide synthase expression and thus, contributes to the production of reactive nitrogen radicals (Oussaief et al. 2011). Interestingly, NF-κB signaling also promotes expression of several antioxidants such as, MnSOD, Zn-SOD and Trx1 (Morgan and Liu 2011), and suggests a role for Sirt-1, in suppressing the ROS production and reducing the antioxidant defense system.

11.2. Sirt-1 in Vascular Remodeling

Sirt-1 is a novel target for therapeutic strategies against vascular remodeling associated diseases. High Levels of Sirt-1 expression have been shown to be protective against cardiac hypertrophy, VSMC proliferation and migration, and vascular inflammation (Li et al. 2011a; Li et al. 2011b; Regnault and Lacolley 2017). Sirt-1 overexpression inhibits neointima formation, a common feature of many vascular diseases, by reducing VSMC proliferation and migration (Li

et al. 2011b). Sirt-1 overexpression also showed to attenuate Ang II-induced VSMC hypertrophy (Li et al. 2011a). Moreover, Sirt-1 activator resveratrol was reported to prevent concentric hypertrophy and cardiac cell dysfunction through the reduction of oxidative stress without decreasing blood pressure in SHR (Thandapilly et al. 2010). Resveratrol also inhibited Ang II-induced cardiomyocyte hypertrophy (Cheng et al. 2004). Our laboratory has also demonstrated that resveratrol treatment attenuates hyperproliferation of VSMC through reduction of superoxide production in SHR (Almajdoob, Hossain, and Anand-Srivastava 2018). However, increased expression of Sirt-1 may be detrimental too. It was reported that overexpression (2.5 to 7.5-fold) of Sirt-1 in transgenic mice attenuated age-related cardiac hypertrophy, apoptosis, cardiac dysfunction, yet 12.5-fold increased expression contributed to cardiac hypertrophy (Alcendor et al. 2007). Moreover, overexpression of Sirt-1 contributed to the increased hypertrophy and pharmacological inhibition of Sirt1 through NAM or sirtinol decreased the size of cardiomyocytes (Alcendor et al. 2004). Another study reported an overexpression of cardiac Sirt-1 and its association with left ventricular hypertrophy in SHR (Li et al. 2009) . Therefore, the role of Sirt-1 overexpression in cardiac hypertrophy has become an important challenge to overcome. Therefore, it is vital to understand the delicate interplay between Sirt-1 and its associated pathways in the cardiovascular system to identify potential therapeutic targets, in near future.

11.3. Sirt-1 in Hypertension

The association between Sirt-1 and hypertension is not fully explored yet. Previously, a study demonstrated that resveratrol mediated activation of Sirt-1 inhibits the renin-angiotensin system by downregulating AT1 receptor signaling, whereas, inhibition of Sirt-1 with nicotinamide upregulates the expression level of AT1 (Miyazaki et al. 2008). However, a recent study showed that Sirt-1 knockout in mice attenuates Ang II-induced hypertension by suppressing the expression of AT1 receptor and protects arterial wall against vascular remodeling (Fry et al. 2015). We have also shown that VSMC from SHR exhibit an enhanced expression of Sirt-1 in comparison with control WKY, and knockdown of Sirt-1 by siRNA decreased the overexpression of Giα-2 protein (unpublished observations)(Hossain, Li, and Anand-Srivastava).These experimental data

collectively suggest that the overexpression of Sirt-1 through augmenting the levels of Giα proteins may play a role in the pathogenesis of hypertension in SHR.

11.3.1. Sirt-1 Inhibitors

In the last years, pharmacological modulation of Sirt-1 has been widely studied and small molecules that can inhibit Sirt-1, showed beneficial effects in cancer therapy (Audrito et al. 2011). Several small molecule inhibitors of Sirt-1 have been used in understanding Sirt-1-associated biological processes. **Sirtinol** is the first discovered Sirtuin inhibitor, inhibits both Sirt-1 and Sirt-2 establishing sirtuins as a potential therapeutic target in cancer therapy (Hwang et al. 2014). **Cambinol** is another inhibitor which effectively blocks both Sirt-1 and Sirt-2 and is used in anti-cancer therapeutic approach (Lugrin et al. 2013; Medda et al. 2009). **EX-527** and its analog **CHIC35** both are indole scaffolds, show most potent selective inhibition against Sirt-1 over other sirtuins (Gertz et al. 2013; Reverchon et al. 2016). **Nicotinamide (NAM)** is a byproduct of Sirt-1 enzymatic reaction **[Figure 11]** has been widely used as an Sirt-1 inhibitor in several studies (Peled et al. 2012). **Suramin** and its analogs are polyanionic urea, also show effective inhibitory effect against Sirt-1 and Sirt-2 (Kalle et al. 2010; Trapp et al. 2007). However, analog **NF675** shows a higher selectivity for Sirt-1 inhibition (Yoon et al. 2014; Rotili et al. 2011). In addition, **Quinoxaline-4bb**, spiro series inhibitors (e.g. **spiro-4c** and **spiro-4e**) can inhibit Sirt- 1 more selectively than hydroxy naphthaldehyde derivatives, sirtinol and cambinol (Rambabu et al. 2013; Ghosh et al. 2017).

11.3.2. Structure and Molecular basis of Selective Sirt-1 inhibitor EX-527

High-throughput screening revealed several indole derivatives as promising Sirt-1 inhibitors. Among them, EX-527 (6-chloro-2,3,4,9-tetrahydro-1H-carbazole-1-carboxamide) or Selisistat **[Figure 12]** is the most potent and selective small molecule that has entered into clinical trials. EX-527 inhibits Sirt-1 ~100-fold more potently with a high selectivity over Sirt-2 and Sirt-3 (Zhao et al. 2013). Kinetic analyses suggest that EX-527 blocks the NAD+ binding sites and prevents the release of deacetylated protein and O-acetyl-ADP-ribose. Thus, it inhibits enzyme-catalyzed deacetylation of Sirt-1. Low molecular weight, cell-permeability and oral bioavailability makes EX-527 a standard chemical tool to study Sirt-1-associated mechanisms and to explore therapeutic uses for Sirt-1 inhibitors.

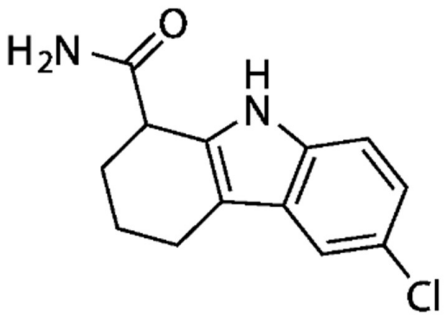

Figure 12: Chemical Structure of EX-527 (6-chloro-2,3,4,9-tetrahydro-1H-carbazole-1-carboxamide) (Selisistat)

11.3.3. EX-527 as potential therapeutic approach

EX-527 has been widely used as a pharmacological inhibitor to study Sirt-1-mediated biological processes and to explore therapeutic uses. EX-527 showed to inhibit Sirt-1 deacetylation activity and increase acetylation of p53 in different cell lines including human mammary epithelial cells (Solomon et al. 2006). In addition, EX-527 prevents cell growth in acute myeloid leukemia by inhibiting deacetylation of p53 (Sasca et al. 2014). It has also been shown that EX-527 is acting as a chemical inducer and promotes differentiation of pluripotent P19 cells into functional neurons (Kim et al. 2016). A recent study suggested neuroprotective potential of EX-527 through necroptosis inhibition in cerebral ischemia–reperfusion injury (Nikseresht, Khodagholi, and Ahmadiani 2019). More intriguingly, EX-527 treatment suppresses deacetylation of mutant huntingtin protein and passed Phase II clinical trials to treat Huntington's disease (Westerberg et al. 2015; Zuccato, Valenza, and Cattaneo 2010). Treatment with EX-527 was also described to protect diabetic nephropathy by lowering blood glucose level and improving kidney morphology in Zucker rats (Kundu et al. 2020)

However, treatment of EX-527 is not always protective, especially when Sirt-1 activation is desired. EX-527 markedly affects atherosclerosis through exacerbating the acetylation of key autophagy machinery (Yang et al. 2017). It also shows to counteract the neuroprotective effects of resveratrol on Parkinson's disease through inhibiting deacetylation of microtubule-associated protein 1 light chain 3 (Guo et al. 2016). Moreover, it was shown to inhibit protective role of Sirt-1 against cardiac cell function and renal proximal tubule cell functions (Jung et al. 2012; Salem et al. 2017).

Hypothesis and Aim

Hypertension is a serious global health issue and a major contributor to the development of cardiovascular disease (Bloch 2016; Fields et al. 2004; Kjeldsen 2018). However, the precise molecular events implicated in the pathological mechanism of hypertension remain poorly characterized. Studies from our laboratory have demonstrated that the overexpression of Giα protein due to enhanced levels of endogenous vasoactive peptides (e.g. Ang II, ET-1), contributes to the pathogenesis of hypertension in SHR (Anand-Srivastava 1996; Hossain et al. 2018; Li et al. 2014; Marcil, Thibault, and Anand-Srivastava 1997). Inhibition of the overexpression of Giα-2 protein by pertussis toxin and anti-sense oligonucleotides attenuated high BP and tachycardia in SHR (Ali El-Basyuni, Li, and Anand-Srivastava 2016; Li and Anand-Srivastava 2002). Further studies showed that treatments with different vasodilators including resveratrol; a polyphenolic molecule, SNP; a nitric oxide donor, and C-ANP4-23; a natriuretic peptide receptor C agonist attenuate increased BP in SHR through the inhibition of overexpression of Giα proteins and nitroxidative stress (Hossain et al. 2018; Li et al. 2014; Sarkar, Li, and Anand-Srivastava 2019).

HDAC Class III has seven members in mammalian namely Sirt-1-7, that regulates gene transcription (Pons et al. 2009). Sirt-1 is a well-studied member of this family which plays a critical role in vascular homeostasis (Satoh, Stein, and Imai 2011; Kupis et al. 2016). Recently, an overexpression of Sirt-1 in hearts and its association with cardiac hypertrophy has been shown in SHR (Li et al. 2009). Another study reported that Sirt-1 knockout mice significantly attenuated Ang II-induced hypertension by suppressing the expression of AT1 receptor suggesting the implication of Sirt-1 in BP regulation (Fry et al. 2015). However, the relationship between Sirt-1, G protein expression and hypertension remain unexplored. Our lab has recently shown that VSMCs from SHR exhibit an overexpression of Sirt-1 as compared to normotensive WKY rats, and knockdown of Sirt-1 by siRNA attenuated the overexpression of Giα-2 protein (unpublished observations) (Hossain, Li, and Anand-Srivastava). Therefore, we hypothesize that the overexpression of Sirt-1 through augmenting the expression of Giα proteins contributes to high BP and tachycardia in SHR. To test this, we will examine the effect of EX-527, a potent inhibitor of Sirt-1 on the development of high BP and tachycardia in SHR and to explore the potential involvement of Giα protein expression, oxidative and nitrosative stress in this process.

CHAPTER 2

Scientific Article

(To Be Submitted to the “Hypertension” Journal)

Inhibition of Sirtuin-1 Overexpression Attenuates Hypertension and Tachycardia in Spontaneously Hypertensive Rats: Role of Giα Protein and Nitro-oxidative Stress

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Abstract:

Sirtuin-1 (Sirt-1), class III histone deacetylase, has been shown to be overexpressed in hearts from spontaneously hypertensive rats (SHR). We recently showed that vascular smooth muscle cells (VSMC) from SHR exhibit enhanced expression of Sirt-1 as compared to age-matched Wistar Kyoto (WKY) rats, which contributes to the upregulation of Giα proteins implicated in the pathogenesis of hypertension. The present study was undertaken to investigate the role of upregulated Sirt-1 expression in the pathogenesis of hypertension in SHR and to explore the underlying molecular mechanisms involved in this response. For this study, a selective inhibitor of Sirt-1, EX-527 (5mg/kg of body weight), was injected intraperitoneally into 8-week-old adult SHR and age-matched WKY rats twice per week for 3 weeks. The blood pressure (BP) and heart rate was measured twice a week by the CODA™ non-invasive tail cuff method. Treatment of SHR with Sirt-1-specific inhibitor, EX-527, attenuated high BP by 76 mmHg and inhibited the augmented heart rate. The overexpression of Sirt-1 and Giα proteins in heart, VSMC and aorta was attenuated to the control levels by Sirt-1 inhibitor. Inhibition of Sirt-1 also attenuated the enhanced levels of superoxide anion, NADPH oxidase activity and the overexpression of NADPH oxidase subunits; Nox2, Nox4 and P47phox proteins in VSMC isolated from EX-527-treated SHR. Furthermore, the decreased levels of endothelial nitric oxide synthase (eNOS) and nitric oxide (NO) and increased levels of peroxynitrite ($ONOO^-$) in VSMC from SHR were also restored to control levels by Sirt-1 inhibitor. These results suggest that the inhibition of overexpression of Sirt-1 through decreasing the enhanced levels of Giα proteins and nitro-oxidative stress attenuates the high BP in SHR. It may thus be suggested that inhibitors of Sirt-1 may have the potential to be used as therapeutic agents in the treatment of cardiovascular complications associated with hypertension.

Key Words: Sirtuin-1, EX-527, Giα protein, SHR, VMSC, Hypertension.

Abbreviations: BP, blood pressure; BW, body weight; eNOS, endothelial nitric oxide synthase; G-proteins, guanine nucleotide regulatory proteins; HDAC, histone deacetylases; NO, nitric oxide; SHR, spontaneously hypertensive rats; Sirt-1, Sirtuin-1; SNP, sodium nitroprusside; VSMC, vascular smooth muscle cells; WKY, Wistar Kyoto.

Introduction

Hypertension (high blood pressure; BP) is a multifactorial disease and one of the factors regulating BP is heterotrimeric guanine nucleotide regulatory proteins (G-proteins)[1-4]. An increased expression of inhibitory G-proteins (Giα proteins) that mediate the inhibition of adenylyl cyclase activity and decrease cAMP levels has been shown to contribute to the pathogenesis of hypertension in spontaneously hypertensive rats (SHR) and DOCA-salt hypertensive rats[5, 6]. This was further supported by the studies showing that the inhibition or inactivation of Giα proteins by pertussis toxin or Giα protein antisense treatment attenuates the development of high BP in SHR [7]. In addition, several vasodilators including resveratrol, sodium nitroprusside (SNP); a nitric oxide donor, and C-ANP$_{4-23}$; a natriuretic peptide receptor C agonist have also been shown to attenuate high BP in SHR through the inhibition of enhanced expression of Giα proteins[8-11].

Histone deacetylases (HDAC) deacetylate lysine residues from histone and non-histone proteins and by their ability to interact with a variety of transcription factors play a key role in regulating the transcription of genes implicated in vascular remodelling[12]. Recent studies have demonstrated that a heightened activation of HDAC notably HDAC4 and HDAC5, is associated with increased proliferation, migration and hypertrophy of VSMC[13-16]. Sirtuin-1 (Sirt-1), an epigenetic regulator is a member of class III HDAC family and is also implicated in a wide range of cellular functions, including modulation of the cell cycle, metabolism, aging, migration and growth of VSMC[17-22]. An overexpression of Sirt-1 in hearts and its relationship with cardiac hypertrophy has been shown in SHR[23]. In addition, Ang II treatment increased BP in wild type mice and this increase was significantly attenuated in Sirt-1 knockout mice[24] suggesting the implication of Sirt-1 in Ang II-induced hypertension. We have recently shown that Sirt-1 is overexpressed in VSMC from SHR and its knockdown by siRNA decreased the overexpression of Giα-2 protein in these cells (unpublished observations)[25]. Taken together, it may be possible that inhibition of overexpressed Sirt-1 may also attenuate high BP in SHR. We undertook the present study to examine the effect of EX-527, a potent and specific inhibitor of Sirt-1[26-28] on high BP in SHR and to explore the underlying molecular mechanisms in this response. We have shown that inhibition of Sirt-1 overexpression by a selective inhibitor, attenuated hypertension and tachycardia in SHR through decreasing the nitro-oxidative stress and the augmented levels of Giα proteins. The study opens

potential avenues towards control of high BP which may help to overcome the prevalence of hypertension in near future.

Materials and Methods

Materials

EX-527 was purchased from Sigma-Aldrich. Western blotting primary antibodies against dynein IC1/2 (sc-13524), eNOS (sc-376751), Giα-2 (sc-13534), Sirt-1 (sc-74504) were purchased from Santa Cruz Biotech (Santa Cruz, CA). However, Nox2/gp91 antibody was purchased from Abcam Inc. (Toronto, ON, Canada). Polyclonal Nox4 antibody was from protein tech. (Manchester, United Kingdom). Polyclonal p47phox antibody from Bioss Inc. (Massachusetts, USA). Secondary antibody goat anti-mouse IgG horseradish peroxidase (HRP) conjugate (sc-2005), donkey anti-goat IgG HRP conjugate (sc-2020), and mouse anti-rabbit IgG HRP conjugate (sc-516252) were purchased from Santa Cruz Biotech (Santa Cruz, CA). Western blotting reagents and enhanced chemiluminescence detection system kits were purchased from Santa Cruz Biotechnologies (Santa Cruz, California, USA).

Animal Treatment

8-week old Male SHR and age-matched normotensive Wistar-Kyoto (WKY) rats were purchased from Charles River Laboratories International Inc. (St-Constant, Quebec, Canada). They were held in a pathogen-free facility on regular rodent chow with free access to water and 12-h light and dark cycles in room temperature. After two days of acclimation, rats were randomly divided in four groups (control WKY rats and SHR without treatment and EX-527- treated WKY and SHR (n=6 in each group). Treatment groups of SHR and WKY were injected intraperitoneally with EX-527 (5mg/kg body weight [BW]) twice per week for three weeks. Control SHR and WKY rats received vehicle alone. At the end of treatment regimen, rats at age 11-week-old (WKY and SHR control and EX-527-treated) after measuring BP, body weight and heart rate, were euthanized by decapitation after CO exposure. Hearts and thoracic aortae were dissected out. Some aortae were used for primary VSMC culture. Hearts and rest of the aortae tissue were immediately frozen in liquid nitrogen and stored at -80°C. All animal procedures used in the present study were approved by the Ethics Committee for experimentation on animals of the University of Montreal (approval 99050). The investigation conforms to the "Guide for the Care and Use of Laboratory Animals" published by the National Institutes of Health. (Guide, NRC 2011).

Cell Culture and Incubation

VSMC from 11-week-old control and EX-527- treated SHR and their age-matched WKY rats were cultured from aortas as described previously[29]. The purity of the cells was checked by immunofluorescence technique using a-actin as described previously[30]. These cells were found to contain high levels of smooth muscle-specific-actin[31]. The cells were plated in 75 cm^2 flasks and incubated at 37°C in 95% air and 5% CO_2 humidified atmosphere in Dulbecco's modified Eagle's medium (DMEM) (with glucose, L-glutamine and sodium bicarbonate) containing 1% antibiotics and 10% heat-inactivated fetal bovine serum (FBS). The cells were passaged upon reaching confluence with 0.5% trypsin containing 0.2% EDTA and utilized between passages 3 and 10. Confluent VSMC were washed three times with PBS and lysed in 200 µl of lysis buffer [25 mM Tris·HCl (pH 7.5), 25 mM NaCl, 1 mM sodium orthovanadate, 10 mM sodium fluoride, 10 mM sodium pyrophosphate, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml aprotinin, 1%Triton X-100, 0.1% sodium dodecyl sulfate (SDS), and 0.5 µg/ml leupeptin] on ice. The cell lysates were centrifuged at 12,000 rpm for 10 min at 4 °C. Protein concentration was measured by Bradford assay [32]and used for the Western blotting.

Preparation of Heart and Aorta Particulate Fraction

Heart and aorta particulate fractions were prepared, as described previously[5, 33]. The frozen hearts and aorta from control and treated WKY and SHR were pulverized into fine powder using a mortar and pestle precooled in liquid nitrogen and homogenized (12 strokes) in a glass homogenizer with the lysis buffer. The homogenate was centrifuged at 12 000g for 15 minutes at 4°C. The supernatants were transferred to a fresh microcentrifuge tube without disturbing the pellet. Protein concentration was determined by Bradford assay [32]. The supernatant was used for Western blotting.

BP and Heart Rate Measurement

BP and heart rate were measured twice per week before and the next day after the treatment with EX-527 by the tail-cuff method without anesthesia, using the CODA standard noninvasive BP system (Kent Scientific Corp), according to the recommendation of American Heart Association[34]. The CODA tail-cuff BP system uses volume pressure recording sensor technology

to measure tail BP. Volume pressure recording is clinically validated and provides 99% correlation with telemetry and direct BP and heart rate measurements. BP was expressed as milligrams of mercury and heart rate as beats per minute.

Western Blotting

Protein expression was determined by Western blotting using specific antibodies as described previously [7, 8]. An equal amount of protein (30 μg) was subjected to 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). After SDS-PAGE, separated proteins were electrophoretically transferred to a nitrocellulose membrane with a semi–dry transfer blot apparatus (Bio-Rad Laboratories) at 15 V for 45 minutes. After transfer, the membranes were washed twice in PBS and were incubated in PBS containing 5% skim milk at room temperature for 1 hour. The blots were then incubated with the specific antibodies: Giα-2, Sirt-1, Nox2, Nox4, p47Phox, eNOS and dynein in PBS containing 0.1% Tween 20 overnight at 4°C. The antigen–antibody complexes were detected by incubating the blots with respective secondary antibodies conjugated with horseradish peroxidase for 1 hour at room temperature. The blots were then washed 3 times with PBS before reacting with enhanced chemiluminescence Western blotting detection reagents (Santa Cruz Biotechnology). The densitometry analysis of immunoblot results was conducted using ImageJ software (http://rsb.info.nih.gov/ij) based on the manufacture's instruction. Briefly, after development, the film was scanned to obtain the digital image. The quantification of immunoblot is based on the intensity (density) of band, which is calculated by area and pixel value of the band. The quantification data are given as ratio between target protein and loading control.

Determination of Superoxide Anion Production and NADPH Oxidase Activity

Basal superoxide anion production in VSMC was measured using the lucigenin-enhanced chemiluminescence method with low concentration (5 μM) of lucigenin as described previously[35]. The cells were washed in oxygenated Kreb–Hepes buffer, scraped and placed in scintillation vials containing lucigenin solution, and the emitted luminescence was measured with a liquid scintillation counter (Wallac 1409; Perkin Elmer Life Science, St Laurent, Quebec,

Canada) for 5 min. The average luminescence value was estimated, the background value was subtracted, and the result was divided by the total weight of proteins in each sample.

After measuring the emitted luminescence for basal superoxide anion production, 0.1 mM β-Nicotinamide adenine dinucleotide 2′-phosphate reduced tetrasodium salt hydrate (NADH, Sigma-Adrich) was added in the vials and the luminescence was measured continuously for 7 min in a liquid scintillation counter (Wallac 1409; PerkinElmer Life Science). NADPH oxidase activity was calculated by subtracting the basal superoxide-induced luminescence from the luminescence value induced by NADH.

Determination of intracellular levels of nitric oxide (NO) and peroxynitrite ($ONOO^-$)

The levels of intracellular NO and $ONOO^-$ in VSMC were measured using intracellular fluorescent probes, diaminofluorescein-2 diacetate (DAF-2DA) and dihydrorhodamine 123 (DHR 123), respectively as described earlier [8, 36]. Confluent VSMC, after washing twice with PBS, were incubated at 37°C for 1 h with both 10 mmol/L DAF-2DA and 10^{-6} mol/L acetylcholine for detecting NO, and with 5×10^{-3} mol/L DHR 123 for detecting $ONOO^-$. Cells were washed twice with PBS, and fluorescence intensities were measured by a Synergy H1 microplate reader with excitation and emission wavelengths at 495 nm and 515 nm for DAF-2DA, and 480 nm and 530 nm for DHR 123, respectively. Changes in fluorescence intensities were expressed as percentages of the values obtained in the WKY group (taken as 100%).

Statistical analysis

Results are the mean ± SEM. Comparisons between groups were made with a one-way analysis of variance (ANOVA) followed by Newman–Keuls test or Bonferroni multiple comparison tests, using GraphPad Prism 5 software. Results were considered statistically significant at values of $P < 0.05$.

Results

In vivo inhibition of Sirt-1 by EX-527 treatment attenuates high BP and tachycardia in SHR

We earlier demonstrated that Sirt-1 is overexpressed in aortic VSMC from SHR and its inhibition by siRNA attenuates the enhanced expression of Giα proteins in these cells (unpublished observations)[25]. Since the enhanced expression of Giα proteins has been shown as a contributing factor in the pathogenesis of hypertension in SHR, it was of interest to investigate if inhibition of Sirt-1 could attenuate high BP in SHR. **Figure 1A** shows the effect of Sirt-1 inhibitor EX-527 on BP profile in SHR and normotensive WKY rats. Mean arterial BP was significantly higher in 8-week-old SHR as compared to age-matched WKY rats (180 ± 6 vs. 119 ± 8 mmHg, $P < 0.001$). Intraperitoneal injection of EX-527 at 5mg/Kg body weight into SHR decreased the BP in a time-dependent manner and after 3 weeks of treatment, the BP was decreased by about 76 mm Hg (BP in control SHR at 11 weeks, 210 ± 5 mmHg, and treated SHR, 134 ± 5 mmHg $P < 0.001$). However, this treatment did not significantly affect the BP in WKY rats (WKY control, 124 ± 3 mmHg and WKY treatment, 112 ± 6 mmHg). In addition, this treatment did not show any adverse effects on the health of the animals because all rats treated with EX-527 maintained or gained weight during the period of the study (weight at 11-week-old control WKY rats, 239 ± 11.2 g; EX-527- treated WKY rats, 224 ± 4.9 g; SHR control, 238 ± 7.5 g; and EX-527-treated SHR, 233 ± 10.8 g).

We also investigated the *in vivo* effect of EX-527 on heart rate in SHR and WKY rats and the results are shown in **Figure 1B**. At age 8 week the heart rate in control SHR was significantly higher than age matched WKY rats (WKY 234 beats/minute (bpm) vs 293 bpm in SHR) and at age 11 week, the heart rate in SHR control was 426 bpm and 253 bpm in WKY rats. EX-527 treatment inhibited the increased heart rate in SHR by about 40% (266 bpm vs 426 bpm). On the other hand, EX-527 did not have any effect on the heart rate in WKY rats.

***In vivo* inhibition of Sirt-1 by EX-527 treatment inhibits overexpression of Sirt-1 in VSMC, Aorta and Heart from SHR**

EX-527 is the highly specific inhibitor of Sirt-1 that inhibits Sirt-1 catalytic activity by interrupting binding of NAD+-derived coproduct [37]. To examine if inhibition by EX-527 could also attenuate the levels of Sirt-1 protein, the effect of EX-527 on the expression of Sirt-1 was investigated. Results shown in **Figure 2** revealed that Sirt-1 is significantly enhanced in VSMC **(Figure 2A),** aorta **(Figure 2B)** and heart **(Figure 2C)** from SHR by about 80%, 60% and 120% respectively as compared to WKY rats and EX-527 treatment almost completely abolished the enhanced expression of Sirt-1 in VSMC whereas in aorta and heart, the attenuation was more pronounced and reached below the control levels. On the other hand, EX-527 did not affect the expression of Sirt-1 in VSMC, aorta and hearts from WKY rats.

***In vivo* inhibition of Sirt-1 by EX-527 treatment inhibits the overexpression of Giα-2 protein in VSMC, Aorta and Heart from SHR**

The inhibition of overexpression of Giα-2 proteins by antisense has been shown to attenuate high BP in SHR[38]. To investigate if EX-527-induced attenuation of high BP is also attributed to the inhibition of enhanced expression of Giα-2 proteins, the effect of EX-527 on the expression of Giα-2 proteins was examined in VSMC, aorta and heart from SHR and WKY rats and the results are shown in **Figure 3**. As reported earlier[38, 39] the levels of Giα-2 proteins are significantly enhanced by about 180% in VSMC **(Figure 3A)** about 90% in aorta **(Figure 3B)** and about 140% in heart **(Figure 3C)** from SHR as compared with WKY rats and EX-527 treatment almost completely restored the enhanced expression of Giα-2 proteins to control levels in VSMC, heart and aorta. On the other hand, EX-527 did not affect the expression of Giα-2 proteins in VSMC, aorta and hearts from WKY rats.

***In vivo* inhibition of Sirt-1 by EX-527 treatment attenuates enhanced oxidative stress in VSMC from SHR**

We earlier showed that enhanced oxidative stress contributes to the overexpression of Giα proteins in SHR [35]. Since, inhibition of Sirt-1 attenuates the overexpression Giα-2 proteins

and high BP, it was of interest to investigate if Sirt-1 inhibition could also attenuate the enhanced oxidative stress in VSMC from SHR. To test this, we examined the effect of EX-527 on the production of superoxide anion (O_2^-) and NADPH oxidase activity in VSMC of SHR and the results are shown in **Figure 4**. As reported earlier [35], the levels of O_2^- were significantly increased by about 200% in VSMC from SHR as compared to WKY rats and treatment with EX-527 attenuated the enhanced levels of O_2^- by about 70% **(Figure 4A)**. In addition, the activity of NADPH oxidase, the main source of O_2^- production in VSMC that was enhanced by about 150% in VSMC from SHR as compared to WKY rats was completely abolished by EX-527 treatment **(Figure 4B)**. On the other hand, this treatment did not have any significant effect on the O_2^- production and NADPH oxidase activity in VSMC from WKY rats.

***In vivo* inhibition of Sirt-1 by EX-527 treatment inhibits overexpression of NADPH oxidase subunits in VSMC from SHR**

To further investigate if the attenuation of enhanced activity of NADPH oxidase activity by Sirt-1 inhibition in VSMC from SHR was attributed to the inhibition of enhanced expression of different subunits of NADPH oxidase, the effect of EX-527 treatment on the expression level of different subunits of NADPH oxidase was examined by Western blotting in VSMC from SHR and WKY rats. Results shown in **Figure 5** indicate that the expression of Nox2 **(Figure 5A)**, Nox4 **(Figure 5B)** and p47phox **(Figure 5C)** was elevated by about 90%, 80% and 70% respectively in SHR as compared to WKY. However, EX-527 treatment attenuated the augmented levels of Nox2, Nox4 and p47phox to control levels without affecting the levels of these proteins in WKY rats.

***In vivo* inhibition of Sirt-1 by EX-527 treatment restores levels of eNOS and NO in SHR**

Earlier studies have shown that the decreased levels of eNOS and NO exhibited by SHR contribute to the development of hypertension [10, 40, 41]. To investigate if EX-527-induced attenuation of high BP is attributed to its ability to restore the decreased levels of eNOS, we determined the expression of eNOS proteins by Western blotting in aorta and heart from SHR and WKY rats. Results shown in **Figure 6** indicate that the expression of eNOS that was significantly decreased by about 60% in aorta **(Figure 6A)** and about 40% in heart **(Figure 6B)** from SHR as compared with WKY rats was restored to control WKY levels by EX-527

treatment. On the other hand, EX-527 treatment did not have any significant effect on the levels of eNOS in aorta and hearts from WKY rats.

We further investigated the effect of EX-527 treatment on the levels of NO in VSMC from control and EX-527- treated SHR and WKY rats and the results are shown in **(Figure 6C).** As reported earlier [8, 39], the intracellular levels of NO were decreased by about 30% in VSMC from SHR as compared to WKY rats and EX-527 treatment restored the decreased levels of NO to control WKY levels. On the other hand, this treatment did not show any significant effect on the NO levels in WKY rats.

***In vivo* inhibition of Sirt-1 by EX-527 treatment attenuates intracellular levels of $ONOO^-$**

We earlier demonstrated that VSMC from SHR exhibit enhanced levels of $ONOO^-$ [8]. Since nitrosative stress due to increased levels of $ONOO^-$ has been shown to contribute to the development of hypertension in SHR [8, 39], it was of interest to investigate if EX-527-induced attenuation of high BP in SHR is also due to its ability to attenuate the increased levels of $ONOO^-$ in SHR. To test this, we determined the levels of $ONOO^-$ in VSMC from control and EX-527 treated SHR and WKY rats and the results are shown in **Figure 7.** As reported earlier [8, 39], the intracellular levels of $ONOO^-$ were increased by about 60% in VSMC from SHR as compared to WKY rats and the treatment with EX-527 attenuated the increased levels to WKY control levels whereas this treatment was without affect in WKY rats.

Discussion

In this present study, we report for the first time that selective inhibition of Sirt-1 by EX-527 attenuates high BP and tachycardia in SHR and is associated with the inhibition of overexpression of Giα-2 protein and nitroxidative stress.

We showed that the intraperitoneal injection of EX-527 into 8-week-old SHR at a concentration of 5mg/kg body weight twice per week significantly attenuated the BP and after 3 weeks of treatment at the age of 11 week-old, the BP was decreased by about 76 mm Hg and reached the same value as that of WKY rats. However, this treatment did not affect the BP in WKY rats. These results suggest that the overexpression of Sirt-1 contributes to high BP in SHR. In this regard, Sirt-1 knockout mice have also been shown to be protective against Ang II-induced high BP[24]. In addition, the role of HDAC in the regulation of BP in different models of hypertensive rats has also been demonstrated[42-45].

The EX-527 treatment also attenuated the overexpression of Sirt-1 in hearts, aorta and VSMC from SHR and suggests the efficacy of EX-527 treatment. Furthermore, EX-527 treatment also attenuated the enhanced expression of Giα-2 proteins in heart, aorta and VSMC from SHR and suggest that EX-527-induced decreased BP in SHR may be attributed to the inhibition of overexpression of Giα-2 proteins that has been shown as a contributing factor in the pathogenesis of hypertension in SHR[38]. In support of this, pertussis toxin (PT), sodium nitroprusside (SNP), natriuretic peptide receptor-C specific agonist, C-ANP$_{4-23}$ and resveratrol-induced attenuation of high BP in SHR was also shown to be associated with the inhibition of the overexpression of Giα-2 proteins[7, 8, 11, 39].

We report for the first time that the inhibition of Sirt-1 by EX-527 treatment attenuates the increased heart rate in SHR and the overexpression of Giα-2 proteins in heart and suggests that EX-527-induced inhibition of augmented heart rate in SHR may be attributed to the attenuation of increased expression of Giα-2 proteins. The implication of enhanced expression of Giα-2 proteins in tachycardia has been demonstrated by the study showing that knock down of Giα-2 proteins by antisense attenuated the increased heart rate in SHR[38].

The implication of enhanced oxidative stress has been shown to contribute to the pathogenesis of several cardiovascular diseases including hypertension[46-48]. We also showed

earlier that resveratrol, C-ANP$_{4-23}$ and SNP-induced attenuation of high BP in SHR was associated with the inhibition of enhanced oxidative stress[8, 10, 11]. Our result showing that EX-527-induced Sirt-1 inhibition attenuates the enhanced production of O_2^-, NADPH oxidase activity and NADPH oxidase subunits Nox2, Nox4 and P47phox suggests that attenuation of high BP by Sirt-1 inhibition may also involve the reduction of oxidative stress. Our results are in contrast with other studies showing that Sirt-1 plays a protective role in regulating the oxidative stress through the activation of various transcription factors including forkhead transcription factors (FOXO), nuclear factor-kappa B (NF-κB) and E2F transcription factor 1(E2F1)[49, 50]. However, Alcendor et al. reported that the moderate overexpression of Sirt-1 in transgenic mice (2.5 fold to 7.5 fold) is protective against oxidative stress whereas high level of Sirt-1 expression (12.5 fold) increases oxidative stress in heart[51]. Thus, the crosstalk between Sirt-1 and oxidative stress needs to be further investigated for the therapeutic use of Sirt-1.

NO and eNOS that catalyzes the formation of NO have been reported to regulate BP[52, 53]. Several studies have demonstrated that hypertensive animals exhibit decreased levels of NO and eNOS[10, 41, 54-56] and contribute to the development of high BP[8]. In addition, eNOS knockout mice were also shown to exhibit high BP with decreased levels of circulating NO[57]. Furthermore, the fact that the inhibition of NO synthase by L-NAME augmented the BP in rats[58] and elevating the intracellular levels of NO by SNP attenuated the development of high BP in SHR[8] supports the implication of decreased levels of NO in hypertension. Our present studies showing that the levels of NO and eNOS are decreased in heart, aorta and VSMC from SHR are consistent with our earlier studies and the studies of other investigators[10, 41, 54-56]. In addition, the results showing that the inhibition of Sirt-1 by EX-527 augmented the reduced levels of eNOS in aorta and heart and NO in VSMC from SHR suggest that attenuation of high BP by EX-527 in SHR may also be due to its ability to enhance the levels of eNOS and NO. In support of this, the augmentation of intracellular levels of NO by NO donors including SNP and nitrite derivatives have been shown to exert antihypertensive effects in SHR and other models of hypertensive rats[59-62].

The enhanced levels of $ONOO^-$ have been shown in VSMC, aorta, mesenteric arteries as well as in kidney from SHR[8, 55, 56]. We recently showed that SNP- as well as C-ANP$_{4-23}$- induced attenuation of high BP in SHR was associated with their ability to decrease the enhanced levels of $ONOO^-$[39]. In the present study, we demonstrate for the first time that inhibition of Sirt-1 by

EX-527 that attenuates high BP also inhibits the enhanced levels of $ONOO^-$ in VSMC from SHR and suggests that EX-527-induced attenuation of high BP in SHR may also be attributed to its ability to inhibit the increased levels of $ONOO^-$ causing nitrosative stress.

In conclusion, we provide the first evidence that EX-527-induced inhibition of Sirt-1 attenuates hypertension in SHR through the inhibition of the exaggerated levels of Giα proteins and nitroxidative stress. These studies also suggest that Sirt-1 inhibitors have the potential to be used as therapeutic agents in the treatment of cardiovascular complications including hypertension.

Perspectives

Hypertension is a multi-factorial chronic disorder affecting over 1 billion people worldwide[63]. The present study demonstrates a role of class III HDAC, Sirt-1 in the overexpression of Giα-2 protein in SHR. It shows for the first time that Sirt-1 inhibition by EX-527 attenuates the high BP through the inhibition of increased expression of Giα proteins and nitro-oxidative stress in SHR, the factors implicated in the pathogenesis of hypertension. Based on these findinge, it can be suggested that EX-527, a potential inhibitor of Sirt-1 may have the potential to be used as therapeutic agent in the treatment of cardiovascular complications including hypertension. Uncontrolled high BP is a major risk factor for CVD morbidity and mortality in US adults[64]. Threfore, effective antihypertensive agents will facilitate controlling hypertension and lessen social and emotional burden.

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Conflicts of Interest

There is no conflict of interest associated with this work.

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Figure Legends

Figure 1*: **In vivo* treatment with EX-527 attenuates high blood pressure (BP) and augmented heart rate in spontaneously hypertensive rats (SHR).** 8-weeks old SHRs and Wistar Kyoto (WKY) were intraperitoneally injected with EX-527 (5 mg/kg body weight) or vehicle twice per week for 3 weeks. BP and heart rate were monitored twice weekly as described in the "Materials and Method" section. Values are represented as mean ± SEM of six rats in each group. *P < 0.05, **P < 0.01 and ***P < 0.001 versus WKY control (CTL), #P < 0.05, ##P < 0.01 and ###P < 0.001 versus SHR control (CTL). n.s is depicted as not significant.

Figure 2: *In vivo* treatment with EX-527 inhibits overexpression of Sirtuin-1 (Sirt-1) in vascular smooth muscle cells (VSMC) (A), aorta (B) and heart (C) from spontaneously hypertensive rats *(*SHR). Whole cell lysates (A) and tissue homogenates (B and C) from SHR and Wistar Kyoto (WKY) rats with or without EX-527 treatment were subjected to Western blotting using antibody against Sirt-1 as described in "Materials and Method". The protein bands were quantified by ImageJ analysis. The results are expressed as ratio of Sirt-1/Dynein of WKY taken as 100%. Values are expressed as mean ± SEM of at least 4 separate experiments. ***P<0.001 vs WKY control (CTL) and ###P<0.001 vs SHR control (CTL).

Figure 3: EX-527 mediated Sirtuin-1 (Sirt-1) inhibition decreases the overexpression of Giα-2 protein in vascular smooth muscle cells (VSMC) (A), aorta (B) and heart (C) from spontaneously hypertensive rats (SHR). Western blotting results of VSMC lysates (A) and tissue homogenates (B and C) from 11-week-old SHRs and Wistar Kyoto (WKY) rats with or without EX-527 treatment by using antibodies against Giα-2. Details are described in "Materials and Method". The protein bands were quantified by ImageJ analysis. The results are expressed as ratio of Giα-2 protein/Dynein of WKY taken as 100%. Values are mean ± SEM of 6 separate experiments. ***P<0.001 vs WKY control (CTL), and ###P<0.001 vs SHR control (CTL).

Figure 4: Sirtuin-1 (Sirt-1) inhibition attenuates superoxide (O_2^-) anion production and NADPH oxidase activity in vascular smooth muscle cells (VSMC) from spontaneously hypertensive rats (SHR). O_2^- production (A) and NADPH oxidase activity (B) were measured in aortic VSMC from 11-week-old SHR and WKY with or without EX-527 treatment by lucigenin-enhanced chemiluminescence method as described in "Materials and Method". Values

are expressed as mean ± SEM of 5 individual experiments using different cell populations. ***P<0.001 vs WKY control (CTL), ###P<0.001 vs SHR control (CTL).

Figure 5: EX-527 treatment reduces overexpression of NADPH oxidase subunits in vascular smooth muscle cells (VSMC) from spontaneously hypertensive rats (SHR). VSMC lysates from 11-week-old SHR and Wistar Kyoto (WKY) rats with or without EX-527 treatment was subjected to Western blotting using specific antibodies against Nox2 (A), Nox4 (B) and P47phox (C) as described in "Materials and Method". Dynein was used as a loading control. The protein bands were quantified by ImageJ analysis. Results are expressed as % of WKY CTL, taken as 100%. Values are means ± SEM of 6 separate experiments using different cell populations. **P<0.01, ***P<0.001 vs WKY CTL, ##P<0.01 ###P<0.001 vs SHR CTL.

Figure 6. EX-527 mediated Sirtuin-1 (Sirt-1) inhibition restores the levels of eNOS protein expression in aorta (A) and heart (B) and increased the intracellular NO levels in aortic vascular smooth muscle cells (VSMC) from spontaneously hypertensive rats (SHR). Aorta (A) and heart (B) tissue homogenates from SHR and Wistar Kyoto (WKY) rats with or without EX-527 treatment was subjected to Western blotting using an anti-eNOS antibody as described in "Materials and Methods". The protein bands were quantified by ImageJ analysis. Confluent VSMC from 11-week-old SHR and WKY rats with or without EX-527 treatment was incubated at 37°C for 1 h with fluorescent probes diaminofluorescein-2 diacetate (DAF-2DA) for the measurement of intracellular NO (C) levels, as described in the "Materials and methods" section. The results are expressed as ratio of eNOS/Dynein of WKY taken as 100%. Values are mean ± SEM of 4 separate experiments. ***P<0.001 vs WKY control (CTL); ###P<0.001 vs SHR CTL.

Figure 7. Effect of Sirtuin-1 (Sirt-1) inhibition on the levels of $ONOO^-$ in aortic vascular smooth muscle cells (VSMC) from spontaneously hypertensive rats (SHR). Confluent VSMC from 11-week-old SHR and WKY rats with or without EX-527 treatment was incubated at 37°C for 1 h with fluorescent probes dihydrorhodamine 123 (DHR123) for the measurement of intracellular $ONOO^-$ levels, as described in the "Materials and methods" section. The results are expressed as percentage of WKY (control) which has been taken as 100%. ***P < 0.001 versus WKY, ###P < 0.001 versus SHR.

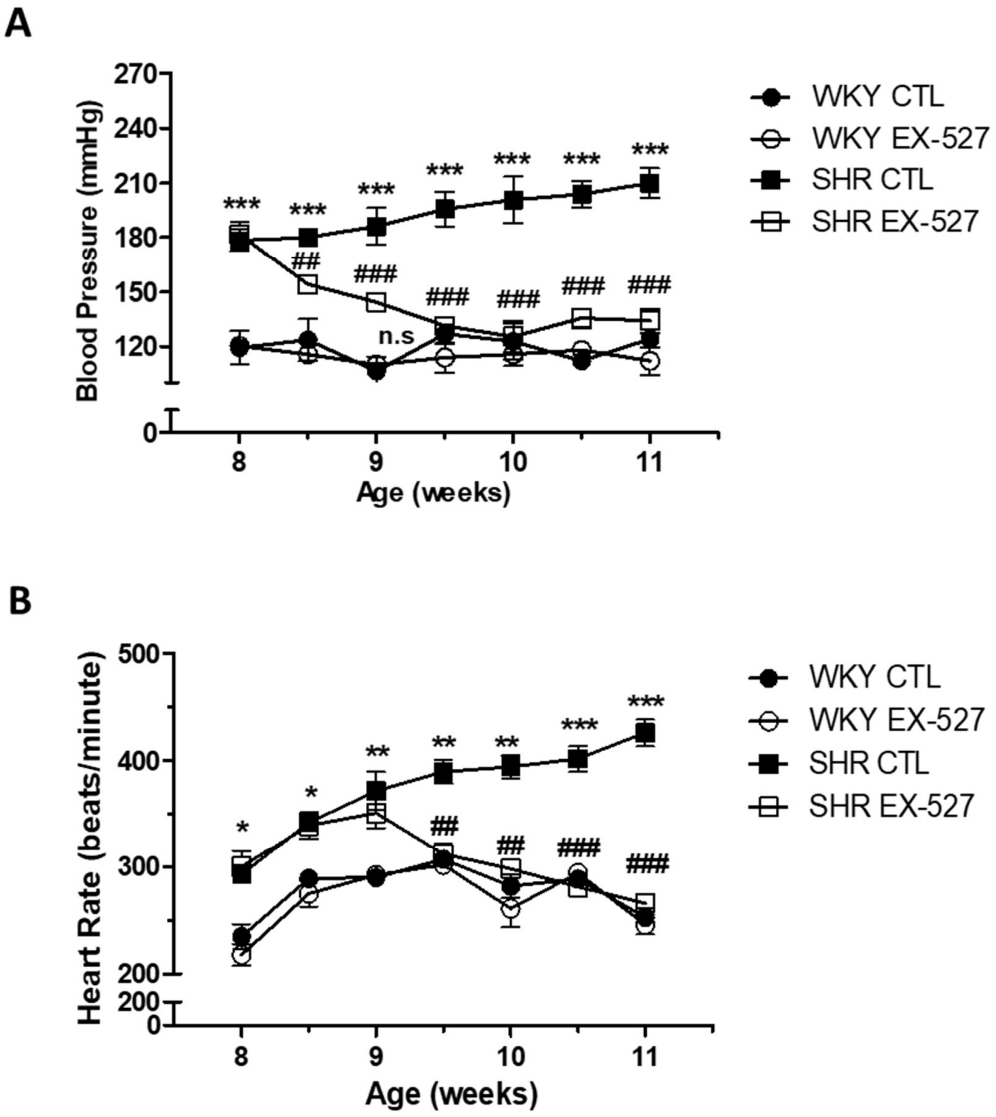
A
Blood Pressure (mmHg)
270
240
210
180
150
120
0
8
9
10
11
Age (weeks)
***
***
***
***
***
***
***
##
###
###
###
###
###
n.s
WKY CTL
WKY EX-527
SHR CTL
SHR EX-527
B
Heart Rate (beats/minute)
500
400
300
200
200
0
8
9
10
11
Age (weeks)
*
*
**
**
**
***
***
##
##
###
###
WKY CTL
WKY EX-527
SHR CTL
SHR EX-527

Figure 1

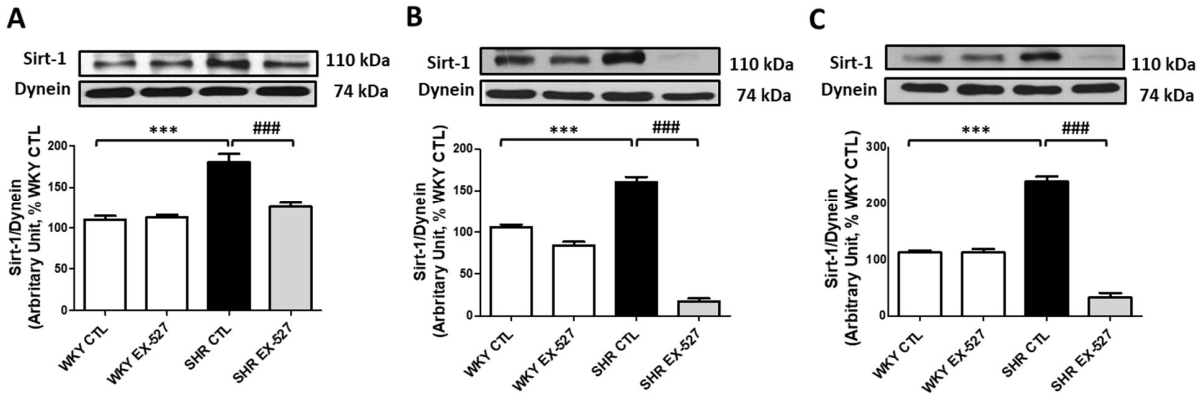
A
Sirt-1
Dynein
110 kDa
74 kDa
Sirt-1/Dynein
(Arbritary Unit, % WKY CTL)
***
###
WKY CTL
WKY EX-527
SHR CTL
SHR EX-527
B
Sirt-1
Dynein
110 kDa
74 kDa
Sirt-1/Dynein
(Arbritary Unit, % WKY CTL)
***
###
WKY CTL
WKY EX-527
SHR CTL
SHR EX-527
C
Sirt-1
Dynein
110 kDa
74 kDa
Sirt-1/Dynein
(Arbritary Unit, % WKY CTL)
***
###
WKY CTL
WKY EX-527
SHR CTL
SHR EX-527

Figure 2

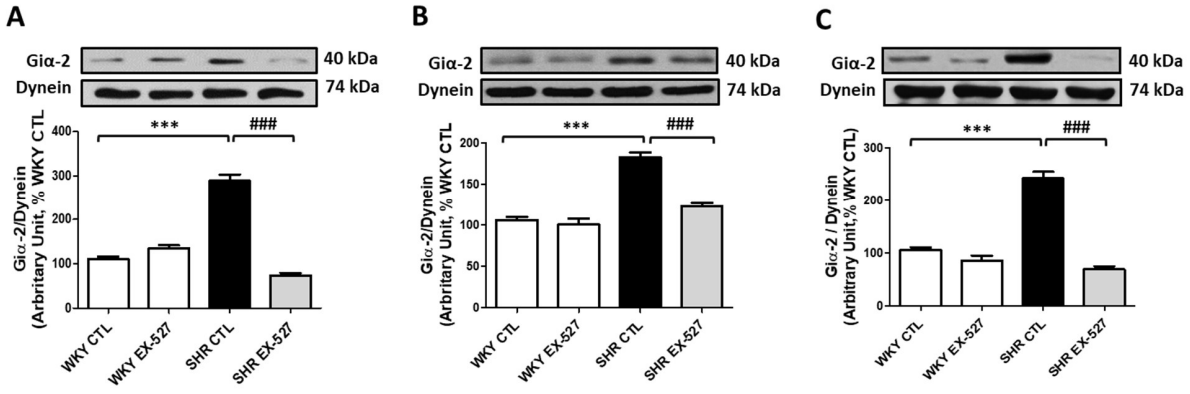
A
Giα-2
Dynein
40 kDa
74 kDa
***
###
Giα-2/Dynein
(Arbritary Unit, % WKY CTL
400
300
200
100
0
WKY CTL
WKY EX-527
SHR CTL
SHR EX-527
B
Giα-2
Dynein
40 kDa
74 kDa
***
###
Giα-2/Dynein
(Arbritary Unit, % WKY CTL
200
150
100
50
0
WKY CTL
WKY EX-527
SHR CTL
SHR EX-527
C
Giα-2
Dynein
40 kDa
74 kDa
***
###
Giα-2 / Dynein
(Arbitrary Unit,% WKY CTL)
300
200
100
0
WKY CTL
WKY EX-527
SHR CTL
SHR EX-527

Figure 3

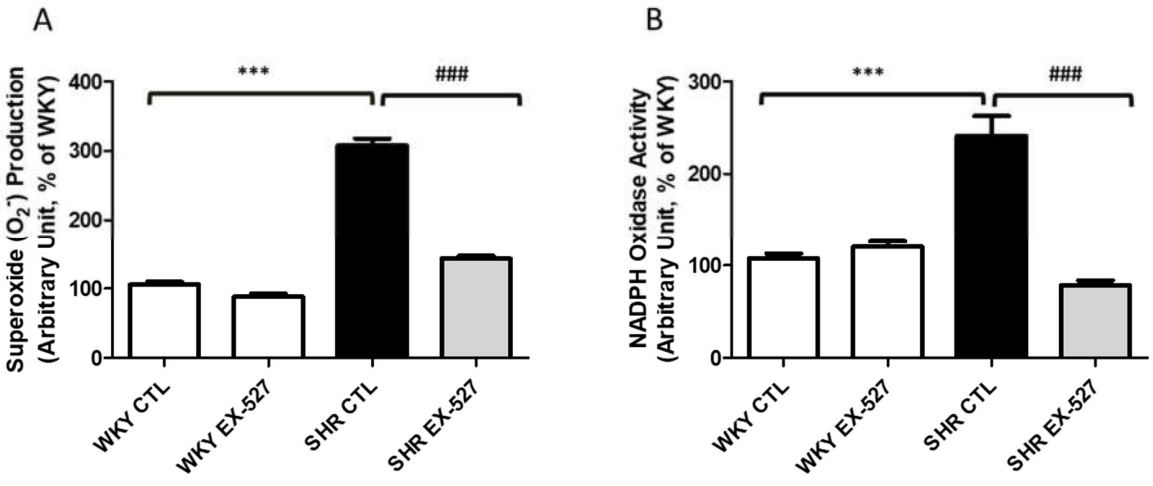
A
Superoxide (O_2^-) Production
(Arbitrary Unit, % of WKY)
400
300
200
100
0
***
###
WKY CTL
WKY EX-527
SHR CTL
SHR EX-527
B
NADPH Oxidase Activity
(Arbitrary Unit, % of WKY)
300
200
100
0
***
###
WKY CTL
WKY EX-527
SHR CTL
SHR EX-527

Figure 4

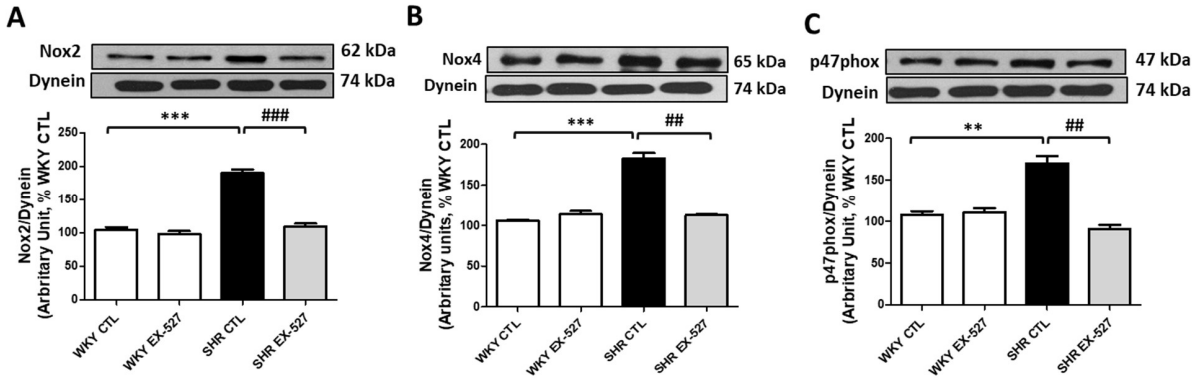
A
Nox2
62 kDa
Dynein
74 kDa
***
###
Nox2/Dynein
(Arbritary Unit, % WKY CTL
250
200
150
100
50
0
WKY CTL
WKY EX-527
SHR CTL
SHR EX-527
B
Nox4
65 kDa
Dynein
74 kDa
***
##
Nox4/Dynein
(Arbritary units, % WKY CTL
200
150
100
50
0
WKY CTL
WKY EX-527
SHR CTL
SHR EX-527
C
p47phox
47 kDa
Dynein
74 kDa
**
##
p47phox/Dynein
(Arbritary Unit, % WKY CTL
200
150
100
50
0
WKY CTL
WKY EX-527
SHR CTL
SHR EX-527

Figure 5

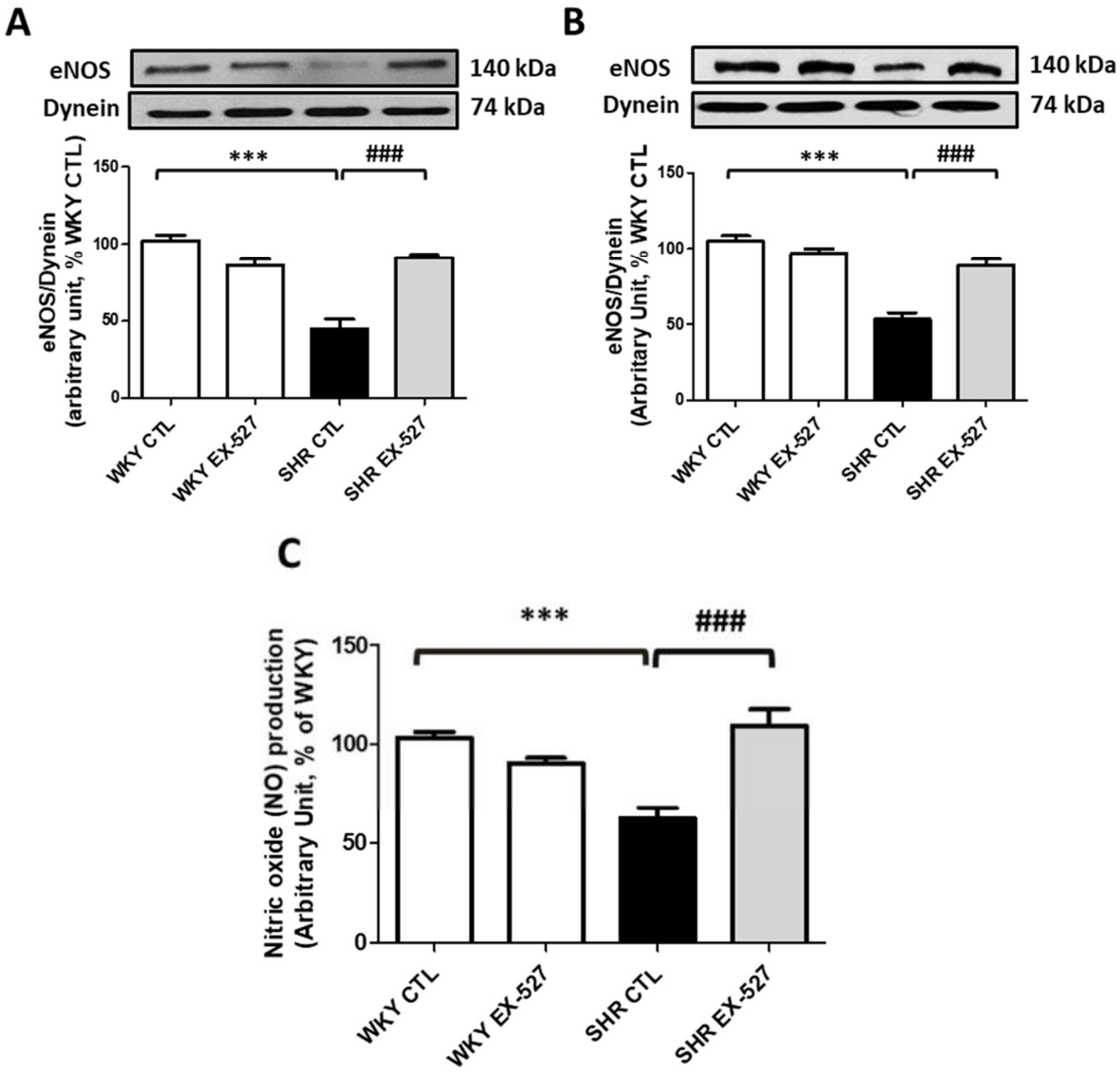
A
eNOS
Dynein
140 kDa
74 kDa
eNOS/Dynein
(arbitrary unit, % WKY CTL)
***
###
150
100
50
0
WKY CTL
WKY EX-527
SHR CTL
SHR EX-527
B
eNOS
Dynein
140 kDa
74 kDa
eNOS/Dynein
(Arbitrary Unit, % WKY CTL
***
###
150
100
50
0
WKY CTL
WKY EX-527
SHR CTL
SHR EX-527
C
***
###
Nitric oxide (NO) production
(Arbitrary Unit, % of WKY)
150
100
50
0
WKY CTL
WKY EX-527
SHR CTL
SHR EX-527

Figure 6

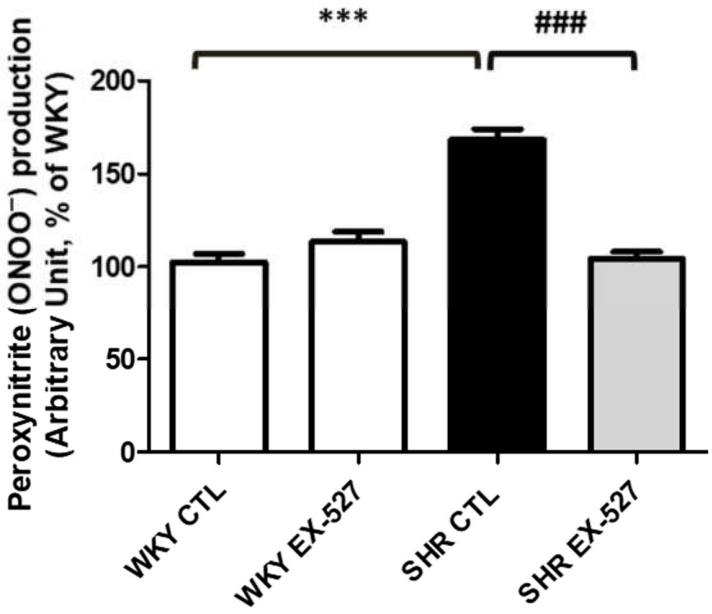
***
###
Peroxynitrite (ONOO⁻) production
(Arbitrary Unit, % of WKY)
200
150
100
50
0
WKY CTL
WKY EX-527
SHR CTL
SHR EX-527

Figure 7

CHAPTER 3

DISCUSSION, CONCLUSION AND FUTURE WORK

Discussion

An overexpression of Giα proteins and associated inhibition of adenylyl cyclase signaling has been shown to contribute to the pathogenesis of hypertension in a variety of animal models including SHR, DOCA-salt hypertensive rats, L-NAME-induced and 1 kidney-1clip (1K1C) hypertensive rats (Anand-Srivastava 1992; Anand-Srivastava, de Champlain, and Thibault 1993; Anand-Srivastava, Picard, and Thibault 1991; Di Fusco and Anand-Srivastava 2000; Ge, Garcia, and Anand-Srivastava 1999). Inhibition of Giα proteins through the injection of pertussis toxin (PT) or using an antisense oligodeoxynucleotide attenuates the development of hypertension and tachycardia in SHR (Li and Anand-Srivastava 2002). In addition, treatment with the vasodilators including resveratrol, SNP; a NO donor, and C-ANP_{4-23}; a natriuretic peptide receptor C agonist have been reported to attenuate high BP in SHR through the inhibition of enhanced expression of Giα proteins (Hossain et al. 2018; Li et al. 2014; Sarkar, Li, and Anand-Srivastava 2019) suggesting the implication of enhanced expression of Gi proteins in the pathogenesis of hypertension.

HDAC enzymes function to remove acetyl groups from lysine residues on the N-terminal tails of histones and non-histone proteins which results in decreased gene transcription (Pons et al. 2009). The overexpression of HDAC is associated with the acceleration of cell proliferation and survival reported in the diseases like cancer and CVD (Eckschlager et al. 2017; Yoon and Eom 2016). Specially, overexpression of HDAC4 and HDAC5 have been shown to contribute to increased proliferation, hypertrophy and cardiac dysfunction (Ginnan et al. 2012; Gordon et al. 2009; Zheng et al. 2019). Therefore, HDAC inhibitors have been targeted as promising therapeutic agents in many diseases.

HDAC inhibitors have been developed as the first successful epigenetic therapy against cancer (Eckschlager et al. 2017). The effects of HDAC inhibition as well as the specific roles of individual HDAC in CVD are still under intense investigation (Habibian and Ferguson 2019). Moreover, HDAC inhibitors have also been targeted as potential intervention for insulin resistance and diabetes mellitus (Chriett et al. 2017; Khan and Jena 2016; Khan, Kumar, and Jena 2016; Sharma and Taliyan 2016). Emerging evidence demonstrates that inhibition of different classes of HDAC ameliorates hypertension in several hypertensive animal models including SHR, DOCA-salt

induced hypertensive rats, Ang II-induced hypertensive rats and pulmonary arterial banding-induced hypertensive rats (Cardinale et al. 2010; Kang et al. 2015; Lee et al. 2013; Zhao et al. 2012).

Sirt-1 is the most studied class III HDAC, has broad physiological effects, including control of gene expression, metabolism and aging (Haigis and Guarente 2006; Michan and Sinclair 2007; Yamamoto, Schoonjans, and Auwerx 2007). Several studies provide evidence that Sirt-1 promotes cell proliferation and overexpression of Sirt-1 leads to tumorigenesis and Sirt-1 inhibitors have been reported to have anticancer activities (Lin and Fang 2013; Hu, Jing, and Lin 2014). The development of small molecules targeting Sirt-1 inhibition has been a focus for many other diseases. Selisistat or EX-527 is the most potent Sirt-1 inhibitor that inhibits Sirt-1 by exploiting the NAD^+ binding of pocket of Sirt-1 structure (Gertz et al. 2013). EX-527 has been undergone phase II clinical trial for the patients with Huntington's Disease (Süssmuth et al. 2015). EX-527 administration was considered safe and well tolerated at dose up to 300 mg (Westerberg et al. 2015).

Recently, Sirt-1 has become also an attractive target molecule in the treatment of hypertension. However, the association between Sirt-1 and BP regulation is still an intriguing topic. Previously Ling Li and colleagues reported an association between Sirt-1 overexpression and cardiac hypertrophy in the heart in SHR (Li et al. 2009). In addition, Fry and colleagues reported that Sirt-1 knockout mice were resistant to the development of Ang II-induced hypertension (Fry et al. 2015). We have recently reported that VSMC from SHR exhibit an enhanced expression of Sirt-1 and inhibition of Sirt-1 by siRNA decreased the overexpression of Giα-2 protein (unpublished observations) (Hossain, Li, and Anand-Srivastava).In this present study, we reported for the first time that inhibition of the Sirt-1 overexpression through intraperitonially injection of EX-527 into 8-week-old SHR at a concentration of 5mg/kg body weight twice per week significantly attenuated the elevated BP and heart rate. However, this treatment did not affect the BP in WKY rats. We also reported that EX-527 treatment inhibited the enhanced expression of Giα-2 proteins in heart, aorta and VSMC from SHR. Previously, we demonstrated that increased expression of Giα-2 protein contributes to the tachycardia in SHR (Ali El-Basyuni, Li, and Anand-Srivastava 2016) and knock down of Giα-2 proteins by antisense attenuated the

increased heart rate (Hossain et al. 2018). These studies suggested that EX-527-induced attenuation of high BP and heart rate in SHR may be attributed to the inhibition of overexpression of Giα-2 proteins.

Increased oxidative stress has been observed as one of the common etiologies in CVD (Cervantes Gracia, Llanas-Cornejo, and Husi 2017; Senoner and Dichtl 2019). Increased production of ROS promotes arterial hypertension by decreasing NO availability which leads to vasoconstriction (Brozovich et al. 2016; Rodrigo, González, and Paoletto 2011). Oxidative stress is the upstream signaling molecule of Giα protein signaling (Bouallegue, Vardatsikos, and Srivastava 2009). VSMC from SHR showed an augmented levels of superoxide anion (O_2^-), NADPH oxidase activity and increased expression of the NADPH oxidase subunits Nox1/Nox2/ Nox4 and p47phox (Gusan and Anand-Srivastava 2013). Moreover, inhibition or knockdown of Giα-2 by resveratrol, C-ANP_{4-23} and SNP treatment is also associated with the attenuation of enhanced oxidative stress in VSMC (Hossain et al. 2018; Li et al. 2014; Sarkar, Li, and Anand-Srivastava 2019) suggesting that attenuation of enhanced oxidative stress is one of the contributing factors in the Giα-2- mediated pathogenesis of hypertension and tachycardia in SHR. Interestingly, we demonstrate that EX-527-induced Sirt-1 inhibition attenuates the enhanced production of superoxide anion O_2^- , NADPH oxidase activity and NADPH oxidase subunits Nox2, Nox4 and P47phox and suggests that attenuation of hypertension through Sirt-1 inhibition may also be associated with the reduction of oxidative stress.

Sirt-1 and oxidative stress both can control each other directly or indirectly (Salminen, Kaarniranta, and Kauppinen 2013; Chong et al. 2012). A considerable body of literature has reported that Sirt-1 activation has a protective role in combatting oxidative stress. For example, Sirt-1 can stimulate the expression of antioxidant enzymes like catalase and manganese superoxide dismutase (MnSOD) through deacetylation of the transcription factor FoxO that has a major role in the cell survival (Alcendor et al. 2007; Daitoku, Sakamaki, and Fukamizu 2011; Giannakou and Partridge 2004). In contrast, Sirt-1 can inhibit transactivation of nuclear factor-kappa B (NF-κB), which is a major immune defense system and can stimulate many antioxidant enzymes (Salminen et al. 2008; Yeung et al. 2004). The enhanced oxidative stress has also been

shown to increase Sirt-1 activity through the activation of c-Jun N-terminal kinase 1 (JNK1), a member of mitogen-activated protein kinases (MAPKs) family that directly phosphorylates Sirt-1 and increases its activity (Nasrin et al. 2009). However, Alcendor et al. demonstrated that the moderate overexpression (2.5-fold to 7.5-fold) of Sirt-1 in transgenic mice could protect against oxidative stress whereas high level of Sirt-1 expression (12.5-fold) increases oxidative stress and induces pathological changes in heart suggesting that after a certain period, overexpression of Sirt-1 could be a factor that stimulates oxidative stress (Alcendor et al. 2007). Thus, further investigations may help to unravel the relationship between Sirt-1 and oxidative stress for the therapeutic use of Sirt-1.

NO and NO forming enzyme eNOS have been implicated in the pathophysiology of hypertension (Wood et al. 2013; Beier et al. 1995; Shesely et al. 1996). Several animal models of hypertension including SHR have been reported to exhibit reduced levels of NO and eNOS that contribute to high BP (Beier et al. 1995; Carlström et al. 2011; Shesely et al. 1996). Moreover, increased production of superoxide anion promotes eNOS uncoupling and reduces NO formation (Luo et al. 2014). Therefore, increasing NO formation via restoration of eNOS offers an important therapeutic strategy for hypertension. Our present study showing that EX-527 treatment augmented the reduced levels of eNOS and intracellular levels of NO in SHR suggests that EX-527-induced attenuation of high BP is attributed to its ability to restore the decreased levels of eNOS and NO. In support of this, our previous study also showed that SNP, a NO donor exerts antihypertensive effects in SHR through the augmentation of intracellular levels of NO (Hossain et al. 2018). NO mediates physiological effects through the activation of soluble guanylate cyclase and formation of cyclic GMP (cGMP) (Denninger and Marletta 1999; Bellamy, Wood, and Garthwaite 2002). Previously, we and others showed that the levels of cGMP were lower in mesenteric arteries and VSMC from SHR compared to WKY rats and were implicated in the development of hypertension in SHR (Archer et al. 1994; Hossain et al. 2018; Fukuda et al. 1991). We showed that SNP-induced attenuation of high BP in SHR is attributed to its ability to increase the levels of cGMP and NO (Hossain et al. 2018). Therefore, it would be interesting to determine the intracellular levels of cGMP in VSMC from EX-527- treated SHR in future to

investigate if EX-527-induced attenuation of high BP in SHR is also attributed to its ability to increase the reduced levels of intracellular cGMP. In addition, our results showing that Sirt-1 inhibition suppressed the increased level of peroxynitrite ($ONOO^-$) from VSMC of SHR suggests that EX-527-induced attenuation of high BP in SHR may also be due to the inhibition of nitrosative stress.

Thus, present study suggests the involvement of epigenetic regulator Sirt-1 in the pathogenesis of hypertension in SHR. The study highlights negative impact of HDAC overexpression in the hypertensive responses in SHR and potential use of HDAC inhibitors in the treatment of hypertension as well. Previously, Zhao et al showed that inhibition of class I HDAC through broad-spectrum inhibitors valproic acid (VPA) and suberoylanilide hydroxamic acid (SAHA) prevented hypoxia-induced pulmonary hypertension in rats (Zhao et al. 2012). Cardinale et al. reported long-term inhibition of class I HDAC through VPA treatment reduces hypertrophic and hypertensive responses in SHR by reducing ROS production and inhibiting AT1 receptor overexpression in the heart (Cardinale et al. 2010). Another study showed that VPA-induced inhibition prevents development of hypertension in deoxycorticosterone acetate (DOCA)–induced hypertensive rats through the attenuation of the transcriptional activity of mineralocorticoid receptor (Lee et al. 2013). Our present study proposed for the first time that inhibition of Sirt-1 overexpression by *in vivo* treatment of EX-527 attenuated hypertension and tachycardia by inhibiting overexpression of Giα protein and nitroxidative stress in SHR. These data support the idea that the role of Sirt-1 should be explored as therapeutic target in hypertension.

The molecular mechanism of hypertension has been shown to be associated with vascular remodeling including hyperproliferation of VSMC. Cultured VSMC from aorta of SHR have been reported to show enhanced proliferation in comparison with normotensive WKY rats (Gusan and Anand-Srivastava 2013). We earlier demonstrated that the levels of several vasoactive peptides including Ang II, ET-1 are increased in hypertension, which contribute to the enhanced proliferation in VSMC from SHR through enhancing oxidative stress, expression level of Giα

proteins, cell cycle proteins and enhanced MAP kinase/PI3 kinase activation (Almajdoob, Hossain, and Anand-Srivastava 2018; Li, Lappas, and Anand-Srivastava 2007). In addition, Fry and colleagues reported the implication of Sirt-1 upregulation in the development of Ang II-induced hypertension (Fry et al. 2015). Moreover, inhibition of Giα proteins overexpression has been shown to attenuate hyperproliferation in VSMC (Gusan and Anand-Srivastava 2013). Therefore, the future studies should be aimed to examine the effect of Sirt-1 inhibition on the activation of MAP kinase/PI3 kinase pathway and proliferation of VSMC from SHR and to explore the underlying molecular mechanisms.

We believe that the results obtained from this study will provide new insights into the underlying epigenetic mechanisms regulating Sirt-1 expression and its role in hypertension and facilitate further research leading to the development of new therapies targeting Sirt-1 for the treatment of hypertension and associated vascular pathology.

Conclusion

The present study has demonstrated the role of Sirt-1 protein, a class III HDAC, in the molecular pathology of hypertension in SHR. VSMC from SHR exhibit an overexpression of Sirt-1 protein and we showed that overexpression of Sirt-1 may be implicated in the pathogenesis of hypertension in SHR. Sirt-1 inhibition already has been suggested as a novel therapeutic strategy for cancer (Kalle et al. 2010; Hu, Jing, and Lin 2014; Yoon et al. 2014; Lara et al. 2009). However, the relationship between Sirt-1 and hypertension in SHR has never been demonstrated. We report for the first time that inhibition of Sirt-1 overexpression by the treatment with a selective inhibitor, EX-527, significantly attenuated high BP in SHR to the control normotensive levels. The overexpression of Giα-2 protein is considered as a contributory factor for high BP and inhibition of Giα-2 protein overexpression is implicated in the attenuation of hypertension in SHR (Anand-Srivastava 1992, 1996; Hossain et al. 2018; Li and Anand-Srivastava 2002; Li et al. 2014; Sarkar, Li, and Anand-Srivastava 2019). We also showed that Sirt-1 inhibition by EX-527 treatment was able to attenuate the expression level of Giα-2 protein and suggests that EX-527-induced attenuation of BP is attributed to its ability to inhibit the overexpression of Giα-2 protein in SHR. In addition, we report that Sirt-1 inhibition also attenuates enhanced oxidative and nitrosative stress in VSMC from SHR which is a major mechanism implicated in hypertension (Lappas, Daou, and Anand-Srivastava 2005). Furthermore, Sirt-1 inhibition also enhanced the bioavailability of NO by augmenting the reduced levels of NO and eNOS in SHR.

In conclusion, we showed for the first time that inhibition of Sirt-1 by EX-527 treatment attenuates hypertension and tachycardia through the inhibition of enhanced nitro-oxidative stress and overexpression of Giα protein in SHR **[Figure 13]**.

Therefore, we believe that overexpression of Sirt-1 may contribute to the pathology of hypertension via enhancing nitrosative stress and expression level of Giα-2 protein in SHR and Sirt-1 inhibitor, EX-527, may have the potential to act as a therapeutic agent against hypertension associated complications.

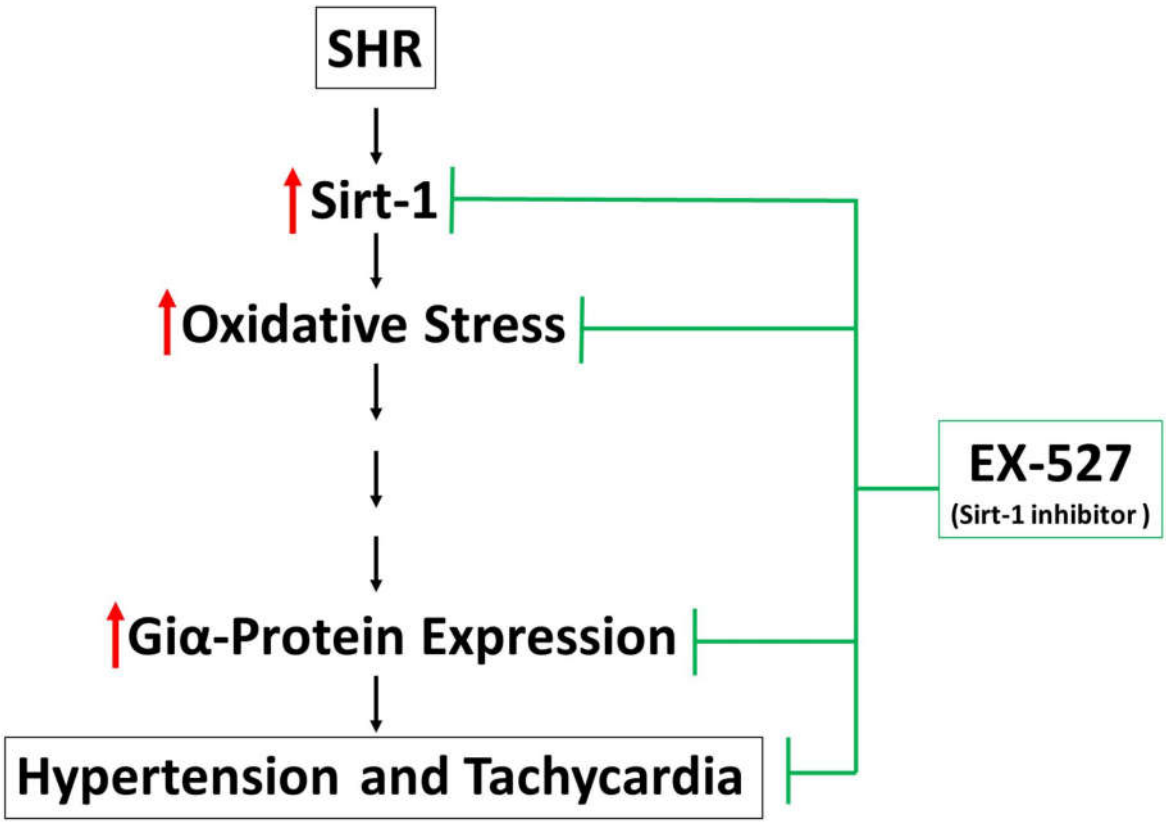

Figure 13: Schematic diagram summarizing the effect of Sirt-1 inhibitor, Ex-527, treatment of Hypertension and Tachycardia in SHR.

Future Work

Our study has elucidated the role of Sirt-1 overexpression in the development of hypertension and tachycardia in SHR. Overexpression of Giα protein is one of the pathological mechanism in hypertension in SHR (Ali El-Basyuni, Li, & Anand-Srivastava, 2016; Marcil, Thibault, & Anand-Srivastava, 1997). We showed that Sirt-1 through augmenting the expression of Giα-2 proteins contribute to high BP in SHR.

Vascular remodeling including abnormal VSMC growth, proliferation, migration, etc. is one of the hallmarks of hypertension (Renna, de Las Heras, & Miatello, 2013). Cultured VSMC from the aorta of SHR exhibit enhanced proliferation and hypertrophy compared with normotensive WKY (Lacolley et al. 2012; Martinez-Quinones et al. 2018). We earlier demonstrated the implication of overexpression of Giα proteins in the vascular remodeling in SHR (Anand-Srivastava & Di Fusco, 2000; Bou Daou, Li, & Anand-Srivastava, 2016).

In addition, we also showed that VSMC from SHR exhibits increased oxidative stress which through the activation of c-Src and growth factor receptors, activates MAP Kinase signaling and increases the expression of Giα and cell cycle proteins and results in hyperproliferation and hypertrophy (Bou Daou, Li, and Anand-Srivastava 2016; Lappas, Daou, and Anand-Srivastava 2005; Sandoval Gomez, Li, and Anand-Srivastava 2011; Sandoval Gomez and Anand-Srivastava 2011). Therefore, the future studies would be aimed to investigate whether Sirt-1 inhibition mitigates increased oxidative stress and signaling pathways implicated in vascular remodeling in SHR and explore the underlying molecular mechanisms.

These studies would further confirm and broaden the scope of this work and help to establish that Sirt-1 inhibitor may serve as a promising therapeutic agent in the treatment of cardiovascular complications associated with hypertension.

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