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Role of the Endocannabinoid System in Cardiac Remodeling

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ROLE OF THE ENDOCANNABINOID SYSTEM IN CARDIAC REMODELING

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ABSTRACT

The endocannabinoid system and CB1 and CB2 cannabinoid receptors have been implicated in cardiac remodeling with CB1 inhibition and CB2 activation reported to be beneficial. However, the role of these receptors in cardiac remodeling secondary to hypertension is not known. Moreover, the functions of the CB1 and CB2 cannabinoid receptors in cardiac ventricular fibroblasts and their downstream fibrotic pathways have not yet been elucidated. This study was designed to determine the temporal myocardial expression pattern of CB1 and CB2 receptors in cardiac remodeling secondary to pressure overload (PO) induced hypertension at 3, 5, 14 and 28 days. The expression of the cannabinoid CB1 and CB2 receptors on human ventricular fibroblasts and the effects of their direct modulation on the fibrotic responses were also investigated. Rat model of pressure overload induced by abdominal aortic constriction was used for the *in vivo* study while the human ventricular fibroblast cell line was used for the *in vitro* study. The presence of CB1 and CB2 receptors was demonstrated on rat fibroblasts in left ventricular cross-sections and on human ventricular fibroblasts for the first time. Myocardial CB1 receptor levels were transiently increased at 3 days before showing decreased expression at the 14 and 28 day time points in the PO groups as compared to sham values. Myocardial CB2 receptor levels were significantly increased at the 3, 5 and 14 day time points in the PO groups as compared to sham values. Thus, the endocannabinoid system was found to be responsive to the pathophysiological changes

occurring in cardiac remodeling secondary to pressure overload with temporal alterations in the expression of cannabinoid receptors. CB1 receptor antagonism on human ventricular fibroblasts was found to decrease their collagen expression and production, thus having an anti-fibrotic effect while CB2 receptor antagonism was found to promote their collagen expression and production, thus having a profibrotic effect. Thus, CB1 antagonism and CB2 agonism seem to be involved in mediating a protective effect against fibrotic responses of the human ventricular fibroblasts. Modulation of these receptors could thus offer an exciting new avenue for development of anti-fibrotic therapies and attenuating adverse remodeling changes.

DEDICATION

I would like to dedicate this dissertation to my parents, my grandparents and my husband for their unconditional love, encouragement and support in all my endeavors.

LIST OF ABBREVIATIONS

AAC	Abdominal aortic constriction
2-AG	2- Arachidonoyl glycerol
AIDS	Acquired immunodeficiency syndrome
AM251	1-(2,4-Dichlorophenyl)-5-(4-iodophenyl)-4-methyl-N-1-piperidinyl-1H-pyrazole-3-carboxamide
AM630	[6-Iodo-2-methyl-1-[2-(4-morpholinyl)ethyl]-1H-indol-3-yl](4-methoxyphenyl)-methanone
AM404	*N*-(4-Hydroxyphenyl)-5*Z*,8*Z*,11*Z*,14*Z*-e icosatetraenamide
Ang II	Angiotensin II
BSA	Bovine serum albumin
CB1	Cannabinoid receptor 1
CB2	Cannabinoid receptor 2
CFs	Cardiac fibroblasts
CP 55940	(-)-*cis*-3-[2-Hydroxy-4-(1,1-dimethylheptyl)phenyl]-*trans*-4-(3-hydroxypropyl)cyclohexanol

DAG	Diacylglycerol
DAPI	4',6-Diamidino-2-phenylindole
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
ECM	Extracellular matrix
ECS	Endocannabinoid system
ERK 1/2	Extracellular-regulated kinases 1 and 2
ET-1	Endothelin-1
FAAH	Fatty acid amide hydrolase
FBS	Fetal bovine serum
FGS	Fibroblast growth serum
HDAC	Histone deacetylase
HRP	Horseradish peroxidase
HSCs	Hepatic stellate cells
HU-210	(6a*R*)-*trans*-3-(1,1-Dimethylheptyl)- 6a,7,10,10a-tetrahydro-1-hydroxy-6,6-dimethyl-6*H*-dibenzo[*b*,*d*]pyran-9-methanol
HVF	Human ventricular fibroblasts
IF	Immunofluorescence
IHC	Immunohistochemistry
i.p.	Intraperitoneal

I/R	Ischemia/reperfusion
JWH-133	(6a*R*,10a*R*)-3-(1,1-Dimethylbutyl)-6a,7,10,10a-tetrahydro-6,6,9-trimethyl-6*H*-dibenzo[*b*,*d*]pyran
LPI	Lysophosphatidylinositol
LV	Left ventricle
MAGL	Monoacylglycerol lipase
MAP	Mean arterial pressure
MAPK	Mitogen activated protein kinase
MCP-1	Monocyte chemoattractant protein-1
MI	Myocardial infarction
MMP	Matrixmetalloproteinases
NAPE	N-arachidonylphosphatidyl ethanolamine
NCS	Neonatal calf serum
NFAT	Nuclear factor of activated T-cells
OMDM-2	(9*Z*)-*N*-[1-((*R*)-4-Hydroxbenzyl)-2-hy droxyethyl]-9-octadecenamide
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline with Triton X-100
PDGF	Platelet derived growth factor
PIC	Protease inhibitor cocktail
PO	Pressure overload

PPAR	Peroxisome proliferator activating receptors
qPCR	Real time polymerase chain reaction
RT	Room temperature
SHR	Spontaneously hypertensive rats
α-SMA	Alpha smooth muscle actin
STAT-3	Signal transducer and activator of transcription-3
TGF-β	Transforming growth factor- beta
TIMP	Tissue inhibitors of metalloproteinases
TNF- α	Tumor necrosis factor- alpha
TRPV1	Transient receptor potential vanilloid subtype 1
WHO	World Health Organization

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INTRODUCTION

Cardiac Remodeling

Remodeling of the heart involves molecular, cellular and interstitial changes, which result in alteration of the structure and function of the ventricles in response to physiological and pathological stimuli. Depending on the etiology, cardiac remodeling consists of myocyte hypertrophy, myocyte necrosis or apoptosis, formation of infarct, changes in left ventricular chamber size, increased collagen production and deposition in the extracellular matrix (ECM) (also termed as fibrosis) and changes in the coronary vasculature. These changes progressively increase the workload of the heart making it susceptible to failure over a period of time. Pathological stimuli, which induce remodeling, include cardiac injury [ischemia, infarction, inflammatory heart muscle disease (myocarditis), idiopathic dilated cardiomyopathy], pressure overload (hypertension, aortic stenosis) and volume overload (valvular regurgitation) (Takano et al., 2003; Fedak et al., 2005a, 2005b).

Pathological remodeling can be divided into two stages: adaptive and maladaptive. Stimuli such as hypertension or aortic stenosis increase the afterload of the heart. Adaptive remodeling helps to maintain a normal cardiac output by the heart against an increased afterload to ensure adequate blood flow to all the organs of the

body. Cardiac output is defined as the amount of blood pumped by the heart in 1 minute (about 5 liters/min, average value for resting adult) (Guyton and Hall, 2010, pg. 229) and afterload is the load or pressure against which the heart has to pump out the blood from the ventricle (Guyton and Hall, 2010, pg. 109). However after the adaptive stage, when the stimuli persist, the hypertrophy and changes in the ECM lead to increased thickness and stiffness of the ventricular walls. This leads to decreased contractility and inability of the left ventricle to maintain the required cardiac output eventually predisposing it to failure over a period of time. This type of remodeling is classified under the maladaptive stage.

Hypertension

Chronic hypertension is one of the major causes of remodeling of the heart. Hypertension is a highly prevalent disorder in today's age with a worldwide incidence of around 40% in adults aged 25 and over, according to a World Health Organization (WHO) report in the year 2008 (Global Health Observatory- Raised Blood Pressure, 2013). In the United States, 1 out of every 3 adults (e.g. about 78 million people) has high blood pressure (Go et al., 2013) and as per statistics from NHANES 2011–12, of those with hypertension, 83 % are aware of their condition, 76 % are under current treatment, 52 % have it controlled and 48 % do not have it controlled [Nwankwo et al., 2013]. The number of people affected by hypertension is increasing every year and the current therapies are only partially beneficial at preventing or reversing the adverse remodeling changes affecting cardiac function (Gustafsson et al., 2010). Thus, there is

a need for improved therapies for preventing or attenuating the pathophysiologic changes in remodeling secondary to hypertension.

Blood pressure is considered to be normal when the systolic over diastolic pressure is 120/80 mm Hg or below. Hypertension is defined as high blood pressure when the systolic over diastolic pressure is consistently above 140/90 mm Hg (Dugdale, 2011). American Heart Association defines the following categories of hypertension:

Table 1: Categories of Hypertension

Blood Pressure Category	**Systolic** mm Hg (upper #)	**Diastolic** mm Hg (lower #)
Normal	less than **120**	less than **80**
Prehypertension	**120** – **139**	**80** – **89**
High Blood Pressure (Hypertension) Stage 1	**140** – **159**	**90** – **99**
High Blood Pressure (Hypertension) Stage 2	**160** or higher	**100** or higher
Hypertensive Crisis (Emergency care needed)	Higher than **180**	Higher than **110**

Source: Understanding Blood Pressure Readings - American Heart Association, 2012

As shown in table 1, blood pressure in the range of systolic over diastolic pressure of 120-139/80-89 mm Hg is considered as prehypertension and individuals in this range are advised to do lifestyle modifications such as reduce salt intake or increase the amount of exercise to prevent redevelopment of hypertension. Multiple consistent readings of systolic over diastolic pressure of 140/90 mm Hg or higher and

160/100 or higher is considered stage I and stage II hypertension, respectively and it has to be therapeutically treated with anti-hypertensive medications and lifestyle modifications to maintain it within the normal range (Understanding Blood Pressure Readings - American Heart Association, 2012). Hypertensive crisis develops when blood pressure increases to systolic/diastolic of 180/110 or higher and the person has to be given emergency medical treatment to control it and prevent severe organ damage (Hypertensive Crisis- American Heart Association, 2012).

Hypertension can be caused secondary to several conditions such as pheochromocytoma, hyperaldosteronism, renal artery stenosis or it can be idiopathic, also known as essential hypertension, where the exact cause is unknown. The majority of the cases of hypertension fall under the idiopathic category and although the exact cause cannot be pinpointed, stress levels, dietary habits and lifestyle are known to be major contributing factors (High Blood Pressure or Hypertension - American Heart Association, 2012)

Chronic hypertension is a persistent stimulus, which increases the afterload of the heart and induces remodeling progressively over a period of time. It can lead to hypertensive heart disease, which primarily involves left ventricular hypertrophy and fibrosis. This can further cause systolic or diastolic dysfunction leading to heart failure. Controlling hypertension with medications such as β- blockers, angiotensin receptor blockers and ACE (angiotensin converting enzyme) inhibitors can slow down the progression of ventricular remodeling or even reverse early remodeling changes and reduce the associated risk of heart failure (Drazner 2011, Lorell and Carabello 2000).

Hypertension Induced Left Ventricular Remodeling

Remodeling of the left ventricle (LV) induced by hypertension causes increase in LV mass and mainly consists of myocyte hypertrophy and changes in the ECM which give rise to fibrosis (Schwartzkopff et al., 1993; Berk et al., 2007; Spinale, 2007). Along with the mechanical stress of the increased afterload, various hormones, growth factors, vasoactive peptides, catecholamines and cytokines contribute to the remodeling process via multiple pathways (Drazner, 2011). These changes will be further described in detail below.

a. Hypertrophy

Myocyte hypertrophy is essentially an increase in the size of the myocytes caused by an increase in the number of sarcomeres, which constitute the contractile machinery of these cells. The transduction of mechanical stress on the heart into a hypertrophic response is not yet completely understood but it is known to involve a range of complex signaling pathways, effects of neurohormonal molecules and activation of fetal genes (Swynghedauw et al., 1999; Lorell and Carabello, 2000). Neurohormones such as angiotensin II (Ang II) and endothelin-1 (ET-1) signal through G-protein coupled receptors, mainly those coupled to $G_{\alpha q}$, and receptor tyrosine kinases to activate the downstream calcium dependent hypertrophic signaling pathways. Some of the known calcium dependent pathways involved in the hypertrophic response are CamKII/HDAC-pathway, the calcineurin-NFAT-pathway and the extracellular-regulated kinases 1 and 2 (ERK1/2). (Kehat and Molkentin, 2010).

Cardiac hypertrophy can be concentric or eccentric. Concentric hypertrophy typically occurs secondary to pressure overload wherein the individual myocytes increase in width with addition of sarcomeres in parallel. This leads to thickening of the chamber wall with either no change or a decrease in chamber size (figure 1). Eccentric hypertrophy usually occurs secondary to volume overload (Lorell and Carabello 2000; Kehat and Molkentin, 2010). Eccentric hypertrophy is a result of lengthening of individual myocytes with addition of sarcomeres in series, which leads to increase in the chamber size. Concentric hypertrophy can lead to diastolic failure while eccentric hypertrophy can lead to systolic failure over a period of time. However, concentric hypertrophy can also progress to eccentric hypertrophy during transition to the maladaptive or decompensated stage of remodeling and further lead to heart failure (Kehat and Molkentin, 2010; Gaasch and Zile, 2011) (figure 1).

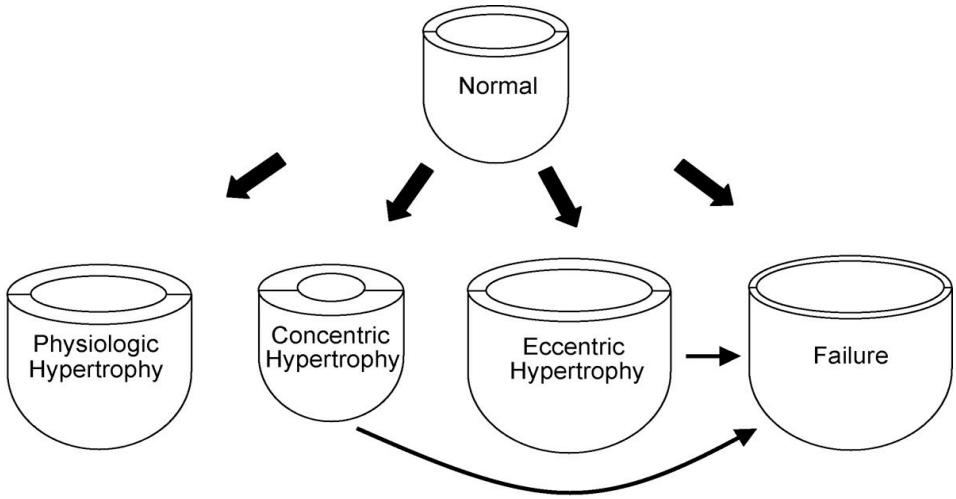


Figure 1: Different types of hypertrophy: Physiologic, concentric and eccentric. Concentric and eccentric can progress to heart failure.

(Kehat and Molkentin, 2010)

b. Changes in the Extracellular matrix

The myocytes are supported by a scaffolding of the extracellular matrix (ECM), which gives a structural support to the myocyte and non myocyte cells of the heart, maintains electrical communication between the myocytes and aids in transmission of mechanical forces across the myocardium during systole and diastole.

b.1. Cardiac Fibroblasts

Cardiac fibroblasts (CFs) are flat spindle-shaped resident cells of the extracellular matrix which are smaller but more numerous than myocytes and make up for the majority of 70% of non-myocyte type of cells in the heart (Porter and Turner, 2009). CFs are the key cells involved in maintaining the homeostasis of the ECM, the structural integrity of the heart and regulating changes in the ECM under disease conditions.

CFs are present in a highly organized manner where they are connected to neighboring fibroblasts as well as myocytes to facilitate transduction of mechanical forces and electrical and signaling mechanisms. CFs are the cells solely responsible for the production and secretion of collagen type 1 and 3, which make up most of the ECM content (Brown et al., 2005; Baudino et al., 2006).

b.2. Collagen Synthesis and Degradation

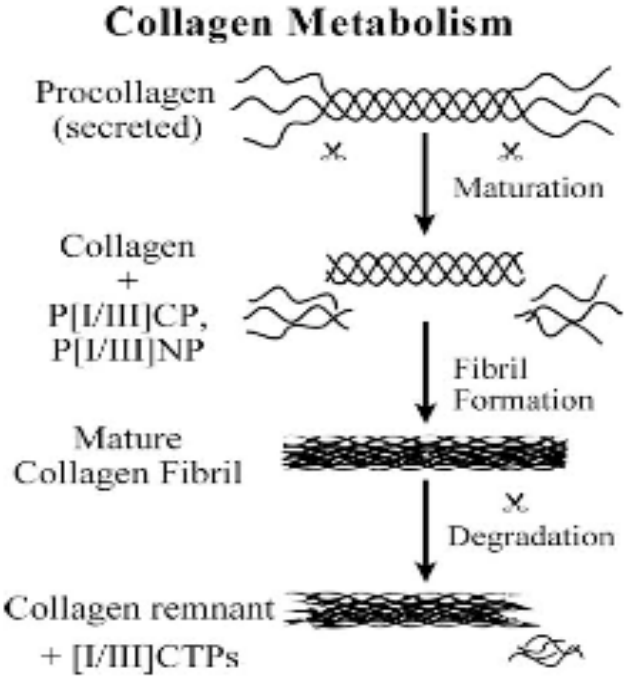


Figure 2: Collagen synthesis and metabolism. Collagen is synthesized as procollagen which undergoes maturation into collagen fibers and bundles. Collagen is degraded by MMPs.

(Brown et al., 2005)

Fibrillar collagen is first synthesized as procollagen inside the cell and transported to the endoplasmic reticulum where hydroxylation of proline and lysine residues occurs. The procollagen is then secreted from the cells in the extracellular space and acted upon by procollagen peptidases to cleave the amino and carboxy terminal propeptides. The mature collagen molecules are then assembled and cross-linked to form collagen

fibrils, which are further linked to form collagen fibers (Brown et al., 2005). Type 1 and 3 collagen constitute the major fibrillar collagen types with type 1 and 3 accounting for about 80% and 10% of the ECM, respectively (Medugorac, 1980; Weber et al., 1989; Speiser et al., 1991; Porter and Turner, 2009). Type 1 collagen provides rigidity to the ECM and has the tensile strength approaching that of steel (Burton, 1954) while type 3 provides elasticity to ensure compliance of the ventricle during diastolic relaxation and filling. CFs also produce the proteolytic enzymes, matrixmetalloproteinases (MMPs), which are a family of over 20 zinc-dependent enzymes involved in degradation of all the proteins of ECM. Degradation of collagen by MMPs leads to release of stable carboxy-terminal telopeptides ([I/III] CTP) (Visse & Nagase, 2003; Raffetto & Khalil, 2008). Tissue inhibitors of metalloproteinases (TIMP) are proteins secreted by the CFs whose most important function is to regulate and inhibit the action of MMPs. MMPs and TIMPs are co-expressed and their functions are well-balanced to regulate the turnover of collagen and maintain the integrity of the ECM (Porter and Turner, 2009; Moore et al., 2012).

b.3 Structure of the Normal Extracellular Matrix and Changes in Hypertension induced Remodeling

The ECM consists of an extensive elastic network of fibrillar collagen, of which collagen type 1 and 3 are the main abundant forms but also small amounts of collagen types 4, 5 and 6. However, the ECM also consists of elastin and fibrillin, fibronectin, laminin, proteoglycans and glycoproteins.

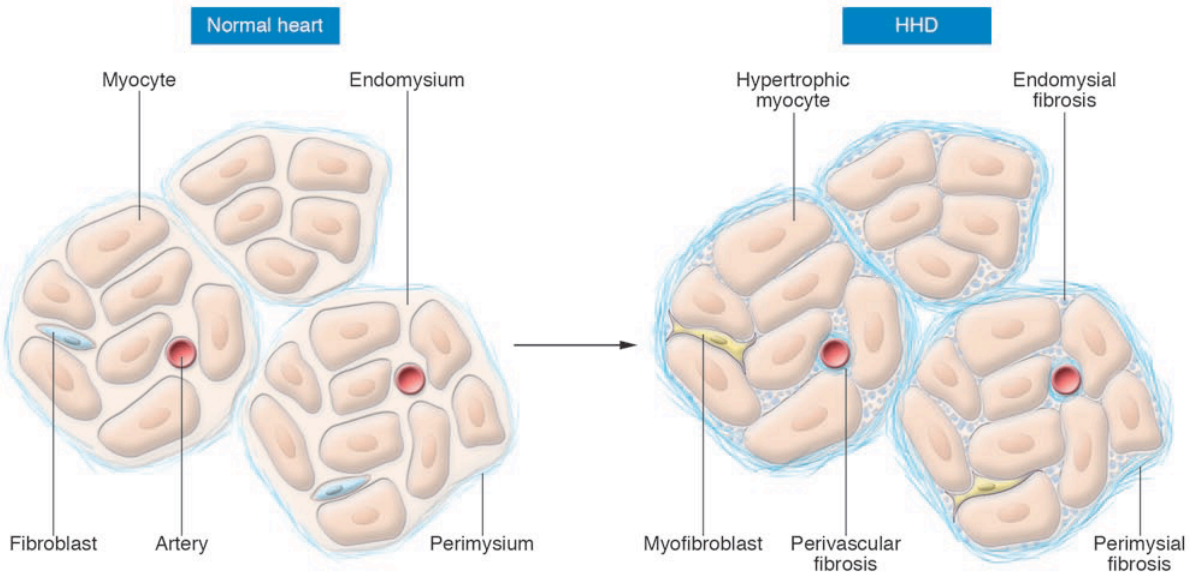


Figure 3: Structure of the ECM in normal heart and hypertensive heart disease (HHD). (Berk et al, 2007)

The endomysium in the ECM is composed of collagen fibers surrounding individual myocytes and those joining adjacent myocytes. The perimysium consists of collagen bundling together groups of myocytes while the epimysium surrounds bundles of perimysium (Berk et al., 2007).

In hypertension induced ECM remodeling, studies have shown perimysial and endomysial fibrosis as well as perivascular fibrosis around the coronary arterial vessels. (Berk et al., 2007). This is caused by a disruption in the equilibrium of the activities of pro and anti-fibrotic mediators involved in a hypertensive state leading to an increased synthesis and deposition of collagen and decreased degradation. The resulting excess in collagen (fibrosis) affects the electrical communication between the myocytes and disrupts their excitation contraction coupling during systole and diastole. It also leads to decreased LV distensibility or compliance, impaired LV filling and diastolic or systolic heart failure.

An abnormal increase in collagen content in postmortem human hearts and myocardial biopsies from hypertensive patients with left ventricular hypertrophy has been demonstrated by several studies (Tanaka et al., 1986; McLenachan and Dargie, 1990; Ciulla et al., 1997; Rossi, 1998; Brilla et al., 2000). A study by Pardo-Mindán and Panizo (1993) found collagen type 1 and 3 to be excessively deposited in the myocardial interstitium and around coronary vasculature in patients having essential hypertension. Increased synthesis of collagen type 1 in patients having hypertension induced heart failure was demonstrated in a study by Querejeta et al. (2004). Mukherjee and Sen (1990) studied alterations in collagen phenotype in spontaneously hypertensive rats (SHR) (an animal model of hypertension and remodeling). The authors concluded that there is an increase in deposition of collagen type 1 during the initial stages of hypertrophy while increased collagen type 3 deposition found at the later stages is associated with a transformation from the compensated to the decompensated state of myocardial dysfunction.

b.4. Functions of CFs in Remodeling

CFs are subjected to cyclic mechanical stretch with every cardiac cycle. But under pathological conditions such as chronic hypertension, the intensity and frequency of the stretch increases leading to changes in the function and phenotype of CFs and resultant composition of the ECM (MacKenna et al., 2000; Gupta and Grande-Allen, 2006). Fibroblasts undergo a phenotypic change to myofibroblasts (having myocyte like properties). Myofibroblasts express alpha smooth muscle actin (α-SMA) and are capable of contractile and migratory functions (Leslie KO et al., 1991; Berk et al., 2007). They have a higher proliferation rate, can migrate to sites of injury such as those caused by ischemia and infarction and cause greater production of collagen and rapid remodeling of the ECM (Arora PD and McCulloch CA, 1994; Campbell SE et al., 1995; Petrov VV et al., 2002). Baicu et al. (2012) found that CFs increased the expression of total collagen 2 weeks after pulmonary artery banding in a feline model of pressure overload and increased production of collagen when the pressure overload became chronic at 4 weeks. Stewart et al. (2010) demonstrated that CFs increased their proliferation and migration after 7 days of abdominal aortic constriction induced pressure overload and this response was concomitant with the onset of hypertrophy and fibrosis within a similar time frame in this rat model of pressure overload.

Increase in mechanical load on the heart induces a profibrotic effect and leads to enhanced gene expression of growth factors and cytokines such as transforming growth factor-β (TGF-β), tumor necrosis factor-α (TNF-α), angiotensin II (Ang II) and endothelin-1 (ET-1) (Weber et al., 1994; Segura et al. 2012). The CFs are stimulated by autocrine and paracrine secretion of these pro-fibrotic factors to produce and deposit

excess collagen in the ECM. The excess collagen deposition leads to fibrosis, which makes the walls of the ventricles thicker and stiffer ultimately affecting cardiac function.

b.5. Role of Profibrotic Factors

Chemokines are involved in immune cell trafficking and help to recruit monocytes, leukocytes and neutrophils to the site of action in response to an injury or inflammatory stimulus (Dobaczewski and Frangogiannis, 2010). MCP-1 (monocyte chemoattractant protein-1), also known as CCL2, belongs to the CC chemokine family and is mainly involved in recruiting monocytes, memory T lymphocytes and natural killer cells (NK) (Valente et al., 1988; Matsushima et al., 1989; Deshmane et al., 2009). MCP-1 is known to be transiently expressed in the endothelium and media of the coronary vasculature in response to hypertension induced by suprarenal abdominal aortic constriction in a pressure overload animal model as seen in a study by Kai et al. (2005). MCP-1 causes chemotaxis of monocytes by extravasation from the circulation. Monocytes are differentiated into macrophages at the tissue specific sites. Macrophages are a source of different cytokines and pro-fibrotic factors such as TNF-α, TGF-β and PDGF (platelet derived growth factor), which stimulate conversion of fibroblasts to myofibroblasts and promote their fibrotic response (Murray and Wynn, 2011). Thus, MCP-1 is known to play an important role in the inflammatory response associated with myocardial infarction (Dobaczewski and Frangogiannis, 2010).

Animal models of pressure overload induced hypertension are known to involve an initial inflammatory phase, which subsides later on (Baumgarten et al., 2002;

Higashiyama et al., 2006). A study by Kuwahara et al. (2004) showed an increase in MCP-1 mRNA expression at 3 days post induction of pressure overload in rats by suprarenal abdominal aortic constriction and the expression was found to subsequently decline. Treatment with an anti-MCP-1 neutralizing antibody was found to reduce macrophage accumulation seen in the transient inflammatory phase, TGF- β induction and myocardial fibrosis but had no effect on cardiac hypertrophy (Kuwahara et al., 2004).

Cardiac fibroblasts have been shown to express MCP-1 (Behr et al., 2000; Manabe et al., 2002). A study by Kermani et al. (1996) demonstrated that MCP-1 can directly stimulate increased secretion of TGF-β_1 by lung fibroblasts, which then further stimulates them in an autocrine loop to increase collagen synthesis. MCP-1 could also possibly increase expression of the profibrotic factor TGF- β by CFs, which can further stimulate them in an autocrine manner to increase collagen production.

The renin angiotensin system is known to be activated in response to hypertension and other pathological stimuli (Timmermans and Smith, 1994; Weber et al., 1994) and Ang II is an important player in the fibrotic process. Ang II has been shown to promote fibrosis via AT1 receptors by directly stimulating proliferation of CFs and production of collagen and other ECM components (Sadoshima and Izumo, 1993; Kawano et al., 2000; Bouzegrhane and Thibault, 2002). Ang II is also known to activate fibroblast activities indirectly by autocrine/paracrine stimulation by increased expression of TGF-β_1 (Dostal, 2001; Schultz et al., 2002).

Transforming growth factor-beta (TGF-β) is an important profibrotic factor in cardiac remodeling. TGF-β expression is up regulated in conditions such as ischemia, infarction or mechanical overload (Manabe et al., 2002). It has been shown to stimulate conversion of fibroblasts to myofibroblasts by inducing expression of α-SMA (Eghbali et al., 1991; Desmoulière et al., 1993). TGF-β is known to directly stimulate the proliferation and migration of CFs as well as increase their collagen synthesis and decrease collagen breakdown in cardiovascular disease states (Villarreal and Dillmann, 1992; Kuwahara et al., 2002; Manabe et al., 2002).

Tumor necrosis factor- α (TNF- α) is another known profibrotic factor playing a role in cardiac remodeling. It is induced in response to hypertension, MI or other pathological stimuli and patients with chronic heart failure have been documented to have increased TNF-α levels in their circulation (Segura et al., 2012). A study by Yokoyama et al. (1999) showed that TNF- α production by neonatal cardiac fibroblasts can be stimulated by Ang II as well as mechanical stretch. Transgenic mice having cardiac restricted overexpression of TNF-α go on to develop dilated heart failure with myocyte apoptosis and overt fibrosis (Bryant et al., 1998).

Introduction to Endocannabinoid system (ECS)

The endocannabinoid system (ECS) has been classically thought of as a neuromodulatory system in the brain constituting the cannabinoid receptors (CB1 and CB2), their endogenous ligands [anandamide and 2-arachidonoyl glycerol (2-AG)] and the enzymes involved in their synthesis and degradation (Gole, 2010, pg.9). The endocannabinoid system is currently under extensive research for the multiple roles it plays in various physiological systems. Its biological effects on the brain have been highly researched due to the widespread use of marijuana obtained from the plant Cannabis sativa. Δ^9-tetrahydrocannabinol (THC) has been found to be the predominant psychoactive constituent of marijuana (Mechoulam and Gaoni, 1967). In the 1980s and early 1990s, studies done with THC led to the discovery of the CB1 receptors in the brain and the CB2 receptors mainly in the periphery. Devane and group first discovered the cannabinoid CB1 receptor in 1988 and it was cloned in 1990 (Matsuda et al, 1990) while the cannabinoid CB2 receptor was cloned in 1993 (Munro et al.).

CB1 receptors have been known to be highly expressed in the brain and to a lesser extent in the peripheral organs, comparatively (Pertwee, 1997; Howlett et al., 2002). The CB2 receptors had been first found to be mainly expressed on different cells of the immune system but have been recently found in the brain, gut, liver, heart and other peripheral organs as well (Onaivi et al., 2006; Pacher and Mechoulam, 2011). Several lines of evidence obtained over the recent years have also suggested the existence of novel cannabinoid receptors (Begg 2005). The GPR55 receptor has been known to be a G-protein coupled novel cannabinoid receptor (Sawzdargo et al., 1999). It has been found to be receptive to the cannabinoid agonist, CP55940, anandamide

and cannabidiol while lysophosphatidylinositol (LPI) and its 2-arachidonoyl glycerol derivative have been considered to be its natural ligands (Ryberg et al., 2007; Okuno and Yokomizo, 2011). Studies have also shown that the peroxisome-proliferator-activating receptors (PPARs) α and γ could be activated by the endocannabinoids at high concentrations (O'Sullivan, 2007) while the transient receptor potential vanilloid subtype 1 (TRPV1) could be activated by anandamide (Starowicz et al., 2007). Research has shown the existence of novel cannabinoid receptors including the putative CB2-like cannabinoid receptor, non-CB1 endothelial receptor coupled to Gi/o and protein kinase G (Mukhopadhyay et al., 2002; Begg et al., 2003) and a putative receptor for anandamide and *R*-(+)-WIN55212 found in several regions of the brain (Howlett et al., 2002).

The cannabinoid receptors are G-protein coupled receptors, mainly linked to the Gi/o-signaling pathways but they have also been found to couple to several pathways including Gs and Gq proteins, mitogen activated protein kinases (MAPK) and protein kinase B (Howlett, 2005). Anandamide has been known to act as a partial agonist and 2-AG as a full agonist at both the CB1 and CB2 receptors (Hillard, 2000). The endocannabinoids are not stored in vesicles like the classical neurotransmitters. They are produced on demand and at the site of action and act as retrograde messengers in the brain. N-arachidonylphosphatidyl ethanolamine (NAPE) has been identified as the phospholipid precursor for the synthesis of anandamide (Liu et al., 2008) by the calcium dependent enzyme NAPE specific phospholipase D (Okamoto et al., 2004) while anandamide has been found to be degraded by the enzyme, fatty acid amide hydrolase (FAAH). Phosphatidylinositol derived diacylglycerol (DAG) has been shown to be the

precursor for the synthesis of 2-AG carried out by the enzymes DAG lipases α and β (Bisogno et al., 1997) while metabolism of 2-AG is known to be carried out by the enzyme monoacylglycerol lipase (MAGL). Studies have shown that the endocannabinoids can also be metabolized by cyclooxygenases, lipoxygenases, and cytochromes P45 (Rouzer and Marnett, 2011). The existence of membrane transporters, which carry out cellular reuptake of both the endocannabinoids, has been postulated as a result of the existence of compounds thought to be cellular uptake inhibitors such as AM404 and OMDM-2 (Beltramo et al., 1997; Hermann et al., 2006; Chicca et al., 2012). The endocannabinoids and CB1 agonists have been known to modulate the release of neurotransmitters such as noradrenaline, dopamine, serotonin and acetylcholine in different brain areas implicated in mood, memory and motor disorders (Szabo and Schlicker, 2005). Owing to the ubiquitous expression of the ECS in different organ systems of the body, it has been implicated in a variety of disorders such as obesity, liver fibrosis/ cirrhosis, cancer, multiple sclerosis, pain, nephropathy as well as a number of cardiovascular disorders (Pacher et al., 2006). Recently, the Food and Drug Administration has approved the use of THC (known as dronabinol, trade name- Marinol; Solvay Pharmaceuticals, Brussels, Belgium) for the treatment of chemotherapy associated nausea and vomiting and for loss of appetite and weight loss associated with acquired immunodeficiency syndrome (AIDS) (Dronabinol: Medline Plus Drug Information- National Library of Medicine, 2010) and its synthetic analogue nabilone (Cesamet; Valeant Pharmaceuticals, Irvine, CA, USA) for treatment of nausea and vomiting caused by chemotherapy (Nabilone: Medline Plus Drug Information - National Library of Medicine, 2010)

ECS in Cardiovascular Physiology and Disease States

The endocannabinoid system was thought to be initially involved in cardiovascular functions with the observation in humans as well as experimental animals of an increase in heart rate following acute use of THC and a decrease in heart rate and blood pressure on chronic use (Rosenkratz, 1974; Benowitz and Jones, 1975). These observed effects of THC were found to be mediated by the cannabinoid receptors (CB1 and CB2), which have only recently been identified in the heart and vasculature in the past decade (Liu et al, 2000; Bonz et al., 2003; Shmist et al., 2006; Mukhopadhyay et al., 2007). Subsequent studies done with exogenously administered endocannabinoids (anandamide and 2-AG) found that both agents: 1) reduce blood pressure via a direct vasodilatory effect; 2) decrease cardiac contractility; and 3) cause bradycardia via inhibition of norepinephrine release by presynaptic CB1 receptors (for review, see Pacher et al., 2005). These effects on blood pressure were found to be tissue and species specific and mediated via various receptors including CB1, vanilloid (TRPV1) and novel non-CB1, CB2 receptors (putative endothelial cannabinoid receptor) (Pacher et al., 2005). Ensuing studies focused on specific cannabinoid receptor type and the role played by this system in various cardiovascular disorders and found that the endocannabinoid system is activated under cardiovascular pathological states.

CB1 receptor antagonism has been shown to be protective against myocardial infarction by reduction in infarct size after treatment with rimonabant, a synthetic CB1 antagonist (Lim et al., 2009). CB1 antagonists exerted protective effects on cardiomyocytes, both in vivo and in vitro, against doxorubicin induced cardiotoxicity (Mukhopadhyay et al., 2007). Further, reduced levels of collagen and profibrotic factors

in CB1 null mice were reported in the model of doxorubicin induced oxidative stress and fibrosis (Mukhopadhyay et al., 2010). A study by Rajesh et al. (2008) showed inhibitory effect of rimonabant on proliferation and migration of human coronary artery smooth muscle cells, which may have a beneficial effect in atherosclerosis and restenosis. Further, CB1 antagonism with rimonabant was found to inhibit macrophage recruitment and atherosclerotic development in mice (Dol-Gleizes et al., 2009). A recent study by Slavic et al. (2013) demonstrated protective effects of rimonabant against fibrosis and cardiac remodeling after myocardial infarction (MI). CB1 antagonism reduced collagen content, downregulated TGF-β_1 expression and improved systolic and diastolic cardiac function after MI in a rat model. There are also some discrepancies in the literature with a few studies showing deleterious effects of CB1 receptor antagonism (Wagner et al., 2003; Mendizabal and Graschinsky, 2007). Also, CB1 activation was shown to have a cardioprotective effect in a mouse model of acute heart failure via inhibition of excessive sympathetic tone (Liao et al., 2012). Reasons for the disparate findings could be due to the complexity of the system with the receptors being linked to numerous signaling pathways, its tonic activation and inactivation under pathological conditions, species and experimental differences. However, to date effects of CB1 activation or receptor blockade in the setting of hypertension induced remodeling remains to be elucidated.

CB2 receptor activation demonstrated cardioprotective effects in a rat model of ischemia/reperfusion (I/R) leading to reduction of infarct size via p38, ERK ½ and PKC (protein kinase C) activation (Lepicier et al. 2003). WIN-55212-2 (non-specific CB1 and CB2 agonist) showed a reduction in reperfusion injury and infarct size as well as decreased activation of macrophages and local generation of cytokines (Di Fillipo et al.,

2004) Further, this beneficial effect was blocked by a specific CB2 antagonist, AM630, thus indicating involvement of CB2 receptors in mediating a cardioprotective effect by reducing recruitment of inflammatory cells in I/R injury (Di Fillipo et al., 2004). Treatment with JWH-133, a specific CB2 agonist, was able to reduce infarct size in a mouse model of I/R when administered shortly before reperfusion and this effect was abolished by the CB2 antagonist, AM630. The cardioprotective effect of CB2 receptors was mediated by three mechanisms, a reduction in superoxide production, which is an early mediator of I/R injury, reduction in neutrophil recruitment and activation of ERK 1/2 and STAT-3 (c) phosphorylation, which is a protective mechanism against myocardial damage after I/R (Montecucco et al., 2009). A study by Defer et al. (2009) demonstrated that CB2 null mice show pervasive myocardial injury, increased myofibroblast differentiation and extensive fibrosis after I/R and the authors concluded that CB2 receptors may play a role in promoting myocyte and fibroblast survival and reduce fibrosis associated with I/R. The synthetic cannabinoid agonist, HU-210 acting via CB2 receptors was found to reduce incidence of ventricular arrhythmias after coronary occlusion and reperfusion (Krylatov et al. 2001). CB2 receptor activation has also been shown to exert an anti-inflammatory effect and attenuate TNF- α induced human coronary endothelial cell activation, expression of MCP-1, transendothelial migration of monocytes and their adhesion to endothelium (Rajesh et al., 2007). It is also known to reduce proliferation and migration of human vascular smooth muscle cells induced by TNF- α (Rajesh et al., 2008). Overall, most of the evidence has demonstrated that CB1 antagonism and CB2 agonism is beneficial in cardiovascular disorders such as myocardial infarction and ischemia, cardiomyopathy and atherosclerosis.

The role played by the ECS in cardiac remodeling secondary to pressure overload has not yet been extensively investigated. The current knowledge base lacks in establishing the effects of pathophysiologic remodeling changes secondary to pressure overload induced hypertension on myocardial expression of cannabinoid CB1 and CB2 receptors and their temporal response to the progressive remodeling changes. We hypothesize that myocardial cannabinoid CB1 and CB2 expression is altered in response to pressure overload induced cardiac remodeling.

The function of CB1 and CB2 cannabinoid receptors in cardiac ventricular fibroblasts and their downstream fibrotic pathways has not yet been studied. Mukhopadhyay et al. (2010) undertook an in vivo study to show CB1 receptors can promote fibrosis in doxorubicin induced cardiomyopathy. Work by Defer et al. (2009) and other groups demonstrated that CB2 receptors can prevent fibrosis in the ischemic heart as well as other tissues such as liver and pancreas (Munoz-Luque et al., 2008; Michalski et al., 2008). Investigating the direct effects of modulation of cannabinoid receptors on cardiac fibroblasts could elucidate a potential anti-fibrotic effect mediated by the endocannabinoid system and thus a novel therapeutic approach. We hypothesize that antagonism of the CB1 receptors expressed on cardiac ventricular fibroblasts would have an antifibrotic effect while antagonism of the CB2 receptors would have a profibrotic effect.

OBJECTIVES

The primary objectives of the present study are:

Aim 1: To determine alterations in cannabinoid CB1 and CB2 receptors in a rat model of cardiac remodeling secondary to pressure overload.

Aim 1.1: To determine levels of collagen and pro-inflammatory factor, MCP-1 (monocyte chemoattractant protein-1) in left ventricular extracts secondary to pressure overload.

Aim 1.2: To demonstrate expression of CB1 and CB2 receptors on fibroblasts in rat left ventricular tissue.

Aim 1.3: To determine the temporal expression pattern of CB1 receptors in the left ventricle at 3, 5, 14 and 28 days post induction of pressure overload.

Aim 1.4: To determine the temporal expression pattern of CB2 receptors in the left ventricle 3, 5, 14 and 28 days post induction of pressure overload.

Aim 2: To determine the effects of CB1 cannabinoid receptor modulation on human ventricular fibroblasts (HVF).

Aim 2.1: To determine expression of CB1 receptors on HVF.

Aim 2.2: To measure changes in total collagen and expression of collagen type 1 and type 3 after treatment of CB1 antagonist, AM251.

Aim 2.3: To determine levels of MCP-1 after treatment of CB1 antagonist, AM251.

Aim 3: To determine the effects of CB2 cannabinoid receptor modulation on human ventricular fibroblasts (HVF).

Aim 3.1: To determine expression of CB2 receptors on HVF

Aim 3.2: To measure changes in total collagen and expression of collagen type 1 and type 3 after treatment of CB2 antagonist, AM630.

Aim 3.3: To determine levels of MCP-1 after treatment of CB2 antagonist, AM630.

MATERIALS AND METHODS

Drugs: 3 compounds, 2- Arachidonoyl glycerol, AM251 and AM630, were used in the in vitro study. Appropriate vehicle controls were used for all the three compounds.

2-Arachidonoyl glycerol (2-AG): 2-AG was obtained from Tocris Bioscience (Minneapolis, MN). It is an endogenous cannabinoid agonist at both CB1 (Ki = 472 nM) and CB2 (Ki = 1400 nM) receptors. The 10 uM solution of 2-AG was made in ethanol.

AM251: AM251 [(1- (2, 4- Dichlorophenyl)- 5- (4- iodophenyl)- 4- methyl- N- 1- piperidinyl- 1H- pyrazole- 3- carboxamide] was purchased from Cayman chemicals (Ann Arbor, MI). It is a potent CB1 receptor antagonist (IC50 = 8 nM, Ki = 7.49 nM) that displays 306-fold selectivity over CB2 receptors. The 10 uM solution of AM251 was made in ethanol and the final concentration of ethanol in the culture media during cell treatments was 1%.

AM630: AM630 {[6- Iodo- 2- methyl- 1- [2- (4- morpholinyl)ethyl]- 1H- indol- 3- yl](4- methoxyphenyl)- methanone} was obtained from Cayman chemicals (Ann Arbor, MI). It

is a CB2 antagonist/inverse agonist (Ki = 31.2 nM) that displays 165-fold selectivity over CB1 receptors. It also behaves as a weak partial/inverse agonist at CB1 receptors with a Ki = 5.2 μM. The 10 uM solution of AM630 used in the study was made in DMSO and the final concentration of DMSO in the culture media during cell treatments was 0.1%.

Animals

9 week old Sprague Dawley rats were used for the study. Rats were housed in a standard temperature and humidity controlled colony room with a 12 h light/12 h dark cycle. Food and water were provided ad libitum. All rats were randomly selected for each group. All the procedures involving animals were approved by the Institutional Animal Care and Use Committee of the University of Mississippi according to the National Institutes of Health Guide for Care and Use of Laboratory Animals.

Abdominal Aortic Constriction Model of Pressure Overload

Pressure overload was induced in rats by abdominal aortic constriction as described in Nagalla et al. (2012). Anesthesia was induced and maintained by using 2% Isoflorane in adult male rats. A sterile heated pad was placed under the animals to maintain their core body temperature. The abdominal skin was sterilized and a midline incision was made under the xiphoid process using surgical scissors. The linea alba was exposed and an incision was made to create a ventral laparotomy. The left abdominal wall of the animal was retracted and maintained in that position using

magnetic clips. The intestines were retracted to one side and the right and left renal arteries arising from the abdominal aorta were identified. A small 21-gauge needle was placed on the part of the abdominal aorta between these two arteries and a size-0 silk suture was tied around the needle and the aorta to partially constrict the aorta to the external diameter of the needle. The needle was quickly removed and the intestines were positioned back in the abdominal cavity. The cut abdominal musculature was closed with an absorbable suture (Chromic 3-0) followed by closure of the skin with skin staples. Sham animals were subjected to the same surgical procedure but without constriction of the abdominal aorta. The animals were then placed in ambient heated units to recover from the anesthesia and surgery.

Assessment of Cardiac Function

Cardiac function was assessed at 14 and 28 days after the induction of pressure overload by abdominal aortic constriction using a Scisense^{TM} admittance catheter (FTS-1912B; Scisense Inc, London Ontario, Canada) as previously described (Pacher et al., 2008; Nagalla et al., 2012). Rats were anesthetized using isofluorane gas contained in an anesthesia chamber. Then the anesthesia was maintained throughout the procedure using sodium pentobarbital at a dose of 10% body weight administered i.p. Animals were placed on a heated pad to maintain their body temperature. An incision was made in the mid neck area and the right carotid artery was exposed after blunt dissection of the thin muscle layer around the throat. The pressure volume conductance catheter was carefully advanced into the left

ventricle (LV) via an incision made in the right carotid artery to measure the LV chamber volume and function. Data were analyzed using iWorx© (Dover, NH) Labscribe Instrument software. Mean Arterial Pressure was calculated as Diastolic pressure + 1/3 (Systolic pressure – Diastolic Pressure).

Tissue isolation

The hearts were extracted from anesthetized rats at the end of measurement of cardiac parameters. The right and left ventricles were identified, separated and weighed. A thin cross-section was cut from the base of the left ventricle and fixed in 4% paraformaldehyde for use in immunohistochemistry. Another section was cut and flash frozen and the non-septal region was used for total collagen measurement by analysis of hydroxyproline content. The remaining portion of the LV containing the apical region was cut into smaller pieces and flash frozen in liquid nitrogen for further biochemical analyses.

Tissue homogenization and protein extraction

The left ventricles from the rats were homogenized and centrifuged and the supernatant was used for biochemical analyses. 100 mg of tissue was weighed from each rat and 800 ul of homogenizing buffer (PBS + 1X PIC) was added to each sample in a glass test-tube. The tissue was homogenized using a hand held homogenizer. 80 ul of cell membrane disruption buffer (10% PBS+Triton X-100) was added to each tube and the samples were vortexed and kept on ice for 30 minutes with intermittent mixing

after every 10-15 minutes. The homogenate was then centrifuged for 30 minutes at 16000 RPM at 4 ^{o}C. The supernatant was collected for protein quantification and other assays.

Protein quantification

Total protein quantification of each sample was done using the DC^{TM} protein assay (BIO-RAD, Hercules CA). Bovine serum albumin was used to make five protein standards (0.1, 0.25, 0.5, 1.0, 1.5 and 2 mg/ml) in 0.1% PBS+Triton X-100 (PBST). The left ventricle tissue samples were diluted 10 times using PBST. 5 ul of standards and samples were added to each well of a 96-well plate in triplicates. Next, 25 ul of activated Reagent A was added to each well followed by 200 ul of Reagent B as per the manufacturer's instructions. The plate was incubated for 15-30 minutes at room temperature (RT) and the absorbance was read at 750 nm on the microplate reader (BioTek, Winooski, VA). The sample dilution was adjusted for in the final calculations of protein concentration (ug/ul) of each sample.

Immunohistochemistry

Immunohistochemistry was performed on rat left ventricular cross-sections fixed in 4% paraformaldehyde to demonstrate expression of CB1 and CB2 receptors on cardiac fibroblasts. LV cross-sections from each sample were paraffin embedded, cut into thin cross-sections and fixed on glass slides (HSRL, Inc. Mount Jackson, VA). The

slides were first deparaffinzed using Xylene (2 X 5 min) and rehydrated in a series of alcohol concentrations in decreasing order (100%, 95%, 70%, 50%) (30-40 sec each). They were thoroughly washed in running water followed by antigen retrieval using citric acid buffer for 20 minutes in a water bath heated to 95-100 ^{o}C. The slides were cooled, washed 2X in water followed by a wash in PBS for 5 minutes each. The sections were then blocked with 5% donkey serum in 1% BSA in PBS for 1 hour at RT. The blocking buffer was decanted after 1 hour and the sections were incubated with either rabbit polyclonal anti-CB1 primary antibody (1:300) in 1% BSA in PBS **or** rabbit polyclonal anti-CB2 primary antibody (1:300) in 1% BSA in PBS at 4 ^{o}C overnight in the refrigerator. Next day, the primary antibody solutions were decanted and the slides were washed in PBS (3 X 5 min) to remove excess primary antibodies. The sections were then incubated with donkey anti-rabbit secondary antibody labeled with Alexafluor 488 for 1 hour at RT. Post incubation of secondary antibody, the slides were washed with PBS (3 X 5 min). The sections were then gently dried and mounted with media containing the nuclear counterstain, DAPI (4',6-Diamidino-2-Phenylindole). The slides were dried overnight and the images of the LV sections were taken using a laser scanning confocal microscope.

Western blotting

Western blotting was carried out to determine expression of CB1 and CB2 receptors using LV extracts and human ventricular fibroblast (HVF) cell extracts. Gel electrophoresis was carried out using 12% Tris-glycine gels. Each lane was loaded with

equal amounts of protein (ug) from LV extracts or cell lysates. The gels were transferred onto a nitrocellulose membrane using a semi-dry transfer apparatus. (Biorad, Hercules, CA). Membranes were blocked with 3% milk in PBS for 3-4 hours at RT and probed with rabbit anti-CB1 polyclonal antibody (1:200, 3% milk in PBS) (Abcam, Cambridge, MA) or rabbit anti-CB2 polyclonal antibody (1:200, 3% milk in PBS) (Cayman Chemicals, Ann Arbor, MI) and rabbit anti-GAPDH polyclonal antibody (Abcam, Cambridge, MA) overnight at 4 ^{o}C. The membranes were washed the next day with 3% milk in PBS (3 X 10 min) and probed with Horse Radish Peroxide tagged goat anti-rabbit polyclonal secondary antibody for 1-2 hours at RT. Following washes with 3% milk in PBS (3 X 10 min), membranes with developed with ECL chemiluminescent reagent (Thermo Scientific, Rockford, IL) and visualized with Versadoc^{TM} imaging apparatus (Bio-rad^{TM}). The expression of CB1 and CB2 receptor proteins was normalized to GAPDH. The positive control used was mouse hippocampus, which is known to have an abundance of CB1 receptors and also known to express CB2 receptors (Pacher et al., 2006).

ELISA (Enzyme Linked Immunosorbent Assay)

Commercially available ELISA kit was used for the analysis of MCP-1 (SA Biosciences, Valencia, CA) in LV extracts and cell culture media. Briefly, standards and diluted samples were added to the 96-well plate and incubated for 2 hours at RT. The plate was washed several times and incubated with the MCP-1 detection antibody solution for 1 hour at RT. Following the washing step, Avidin-HRP was added to the plate and incubated for 30 min at RT. The plate was then incubated with the

development solution for 15 minutes followed by addition of the stop solution. The plate was read at 450nm on the microplate reader (BioTek, Winooski, VA).

Hydroxyproline Assessment of Total Collagen Concentration

A portion of left ventricular tissue (50 mg) was dried at 60 ^{o}C in an oven for 20-24 hours. For the in vitro study, 1 ml of cell culture media from all the samples was dried at 60 ^{o}C for 20-24 hours. Dried samples were then hydrolyzed in 6 N hydrochloric acid at 110 ^{o}C for 20-24 hours. After hydrolysis, samples were decolorized with activated charcoal and filtered to remove the charcoal. The filtrate was then lyophilized by vacuum rotary evaporator. The residue was dissolved in citrate buffer and hydroxyproline concentration was determined using the method described by Woessner (1961).

Cell culture

Human ventricular fibroblasts (HVF) (ScienCell^{TM}, Carlsbad, CA) were grown in DMEM (Cellgro, Manassas, VA) with 10% fetal bovine serum (FBS), 5% neonatal calf serum (NCS) (Atlanta Biologicals, Flowery Branch, GA), 1X fibroblast growth serum (FGS) (Sciencell^{TM}, Carlsbad, CA), 5ug/ml Plasmocin^{TM} Prophylactic (Invivogen, San Diego, CA) and 1X Antibiotic – Antimycotic solution (Santa Cruz Biotechnology Inc., Dallas, TX). For all the studies except those for qPCR (Real Time Polymerase Chain Reaction), cells were grown in 100mm cell culture dishes. Cells were grown to

confluence and serum starved for 24 hours prior to treatment. The treatments with the cannabinoid compounds were carried out for 48 hours with re-dosing of the drug/s after 24 hours. Media was collected for determination of secreted collagen and MCP-1. Cells were scraped after addition of cell lysis buffer (Cell Signaling Technology, Danvers, MA), sonicated and centrifuged at 16000 RPM for 30 min at 4 ^{o}C. The supernatant of the cell extracts was used for further analyses. For qPCR studies, cells were grown in 60 mm culture dishes, serum starved for 24 hours and treated with the cannabinoid compounds for 24 hours. The media was discarded, cells were washed with PBS and the RNA was isolated using the RNeasy Mini kit (Qiagen, Valencia, CA).

Immunofluorescence (IF): Human ventricular fibroblasts (HVF)

HVF were cultured and grown on 8 chamber slides. Cells were fixed with 4% paraformaldehyde, washed with PBS and blocked with 1% BSA in PBS for 1 hour. Rabbit anti-CB1 and rabbit anti-CB2 antibodies (both at a concentration of 1:100 in 1% BSA in PBS) (Thermo Scientific, Rockford, IL) were added to the chambers for 1 hour at RT. Cells were washed with PBS (2 X 10 min) and incubated with goat anti-rabbit secondary antibody labeled with Alexafluor 488 (1:500 in 1% BSA in PBS) for 1 hour at RT. Following the incubation with secondary antibody, cells were washed 3X with PBS, nuclei were counterstained with DAPI and slides were mounted with anti-fade medium (Invitrogen, Grand Island, NY). The cells were visualized with a Nikon Eclipse 90i fluorescence microscope.

Real Time Polymerase Chain Reaction (qPCR)

qPCR was carried out to determine mRNA expression of collagen I and III in HVF. The RNA was isolated from the HVF with the RNeasy Mini kit (Qiagen, Valencia, CA). About 200 ng of RNA was reverse transcribed into cDNA using the iScript cDNA synthesis kit (Biorad, Hercules, CA) from each sample followed by qPCR on the Biorad CFX Connect qPCR Detection System. FAM labeled TaqMan collagen 1A1 (ref no. Hs00164004_m1) and collagen 3A1 (ref no. Hs00943809_m1) primers and VIC labeled TaqMan GAPDH primer (ref no. Hs02758991_g1) were purchased from Applied Biosystems (Grand Island, NY). The changes in mRNA expression were calculated by the ΔΔCt method and normalized to the appropriate vehicle controls.

Statistical analyses

All the results were statistically evaluated using Graphpad Prism Version 6.0 (GraphPad Software, San Diego CA, USA). The values are represented as mean ± SEM. All data with comparison of two groups were analyzed by Student's T-test and those with three or more groups were analyzed by one way analysis of variance (ANOVA) followed by Dunnett's or Bonferroni's post hoc test as specified to determine statistical significance at $p \leq 0.05$.

AIM 1

Aim 1: To determine alterations in cannabinoid CB1 and CB2 receptors in a rat model of cardiac remodeling secondary to pressure overload.

Aim 1.1: To determine levels of collagen and pro-inflammatory factor, MCP-1 (monocyte chemoattractant protein-1) in left ventricular tissue extracts secondary to pressure overload.

Aim 1.2: To demonstrate expression of CB1 and CB2 receptors on fibroblasts in rat left ventricular tissue.

Aim 1.3: To determine the temporal expression pattern of CB1 receptors in the left ventricle at 3, 5, 14 and 28 days post induction of pressure overload.

Aim 1.4: To determine the temporal expression pattern of CB2 receptors in the left ventricle 3, 5, 14 and 28 days post induction of pressure overload.

Rationale:

Hypertension is a rampant disorder afflicting 1 in 3 adults in the United States (Go et al., 2013). It is a leading cause of cardiac remodeling and predisposes the heart to an increased risk of failure. Research in the past decade has shown the existence of CB1

and CB2 receptors in the heart (Bonz et al., 2003; Weis et al., 2010). Studies have implicated the endocannabinoid system in cardiac remodeling associated with ischemia and cardiomyopathy, with CB1 receptor antagonists and CB2 receptor agonists linked to beneficial effects (Lepicier et al., 2003; Defer et al., 2009; Lim et al., 2009; Mukhopadhyay et al., 2010) However, the role of the endocannabinoid system remains yet to be actively investigated in cardiac remodeling induced by chronic hypertension. It is not yet known how the pathophysiologic remodeling changes secondary to pressure overload induced hypertension affect the myocardial expression of cannabinoid CB1 and CB2 receptors and whether they are altered in a temporal manner. We hypothesize that myocardial cannabinoid CB1 and CB2 expression is altered temporally in response to pressure overload induced cardiac remodeling.

Experimental approach:

The animal model of pressure overload (PO) induced by abdominal aortic constriction is a well-established model of hypertension, which induces extensive remodeling of the myocytes and extracellular matrix (Ruetten et al., 2005; Kamogawaa et al., 2001). Unlike other cardiac models of ischemia associated with large oxidative stress, prolonged infiltration of inflammatory cells and loss of myocytes, this model of pressure overload only leads to immune cell influx in the first 3-5 days (Higashiyama et al., 2007).

For this study, pressure overload (PO) was induced in 9-week old male Sprague Dawley rats by abdominal aortic constriction. Sham animals (controls) underwent the same surgical protocol except with no constriction of the abdominal aorta. 8 groups of

rats (n=6/group) were used, 1) 3-day sham, 2) 5-day sham, 3) 14-day sham, 4) 28-day sham 5) 3-day PO, 6) 5-day PO, 7) 14-day PO and 8) 28-day PO, to compare each PO group to their respective age and time matched controls.

Cardiac functional parameters were assessed at 14 and 28 days post surgery for the specific groups using a high fidelity pressure volume catheter in anesthetized rats. Left ventricular tissues were isolated, weighed and snap frozen from all the animals. A thin cross-section was cut from each left ventricle and formalin fixed. The LV cross-sections were then paraffin embedded and cut into thin sections to be used for immunohistochemistry (IHC). IHC was performed to demonstrate expression of CB1 and CB2 receptors on rat LV fibroblasts. Western blotting was utilized to determine temporal changes in expression of CB1 and CB2 receptors in the left ventricular tissue extracts at the 3, 5, 14 and 28 day time points. The levels of MCP-1 in 3 and 5 day time point animals were determined by ELISA. Total collagen content in left ventricular tissues was measured by determination of hydroxyproline concentration in 14 and 28-day animals.

RESULTS

Table 2: Analysis of cardiac parameters at 14 and 28 days in rat pressure overload (PO) model.

	Body Wt (Gms)	LV Wt (Mgs)	LV/ Body Wt Index	MAP (mmHg)
14 Day				
Sham	335 ± 5	752 ± 47	2.2 ± 0.1	115 ± 7
PO	296 ± 8 *	839 ± 27 *	2.9 ± 0.2 *	155 ± 4 *
28 Day				
Sham	362 ± 14	792 ± 47	2.2 ± 0.1	121 ± 5
PO	353 ± 18	971 ± 80 *	2.8 ± 0.1*	158 ± 11 *

Table 2: Comparison of body weights, LV (left ventricle) weights, LV/body weight indices and mean arterial pressure (MAP) at 14 and 28 day time points in sham and PO rats. Values represented as mean ± standard error where *$p < 0.05$. (Student's T-test)

Table 2 shows a significant decrease in body weight of rats undergoing pressure overload surgery at 14 days but the weight was normalized by 28 days as compared to sham values. The left ventricular mass and LV/body weight index (a classic indicator of left ventricular hypertrophy) were significantly increased at both 14 and 28 day time points in PO rats as compared to sham values. However, the significance value of LV/body weight index at the 14 day time point could have been slightly overestimated due to the decrease in body weight of this group. Relative to sham values, the mean arterial pressure (MAP) was also significantly increased at both 14 and 28 day time points in PO rats (Table 2). These cardiac parameters demonstrate cardiac remodeling changes secondary to pressure overload induced hypertension.

Figure 4: MCP-1 (pg/ml) levels in myocardial tissue from sham and PO rats at 3 and 5 day time points.

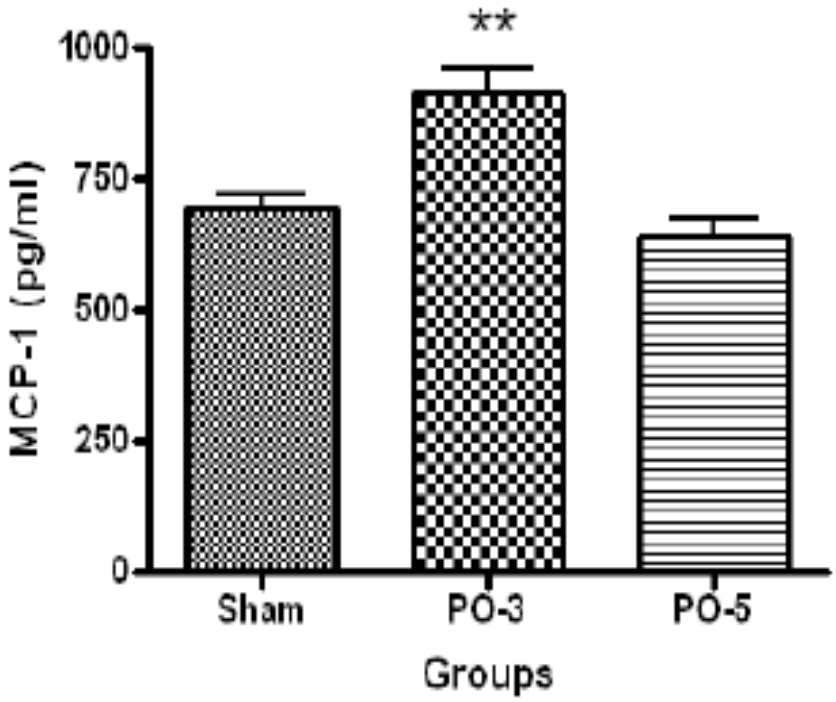


Figure 4: Levels of MCP-1 (pg/ml) at 3 and 5 day time points in sham and PO rat myocardial tissue. Data are represented as mean ± SEM, n=6 mice per group where **p ≤ 0.01 as compared to sham control.

The levels of the inflammatory chemokine MCP-1 were determined by ELISA. MCP-1 levels were found to be significantly elevated in rat left ventricular tissue extracts at the 3 day time point post induction of pressure overload as compared to sham values (figure 4). The elevation seen in MCP-1 was back to control level by day 5.

Figure 5: Total collagen level in rat myocardium in sham and 14 and 28 day PO rats.

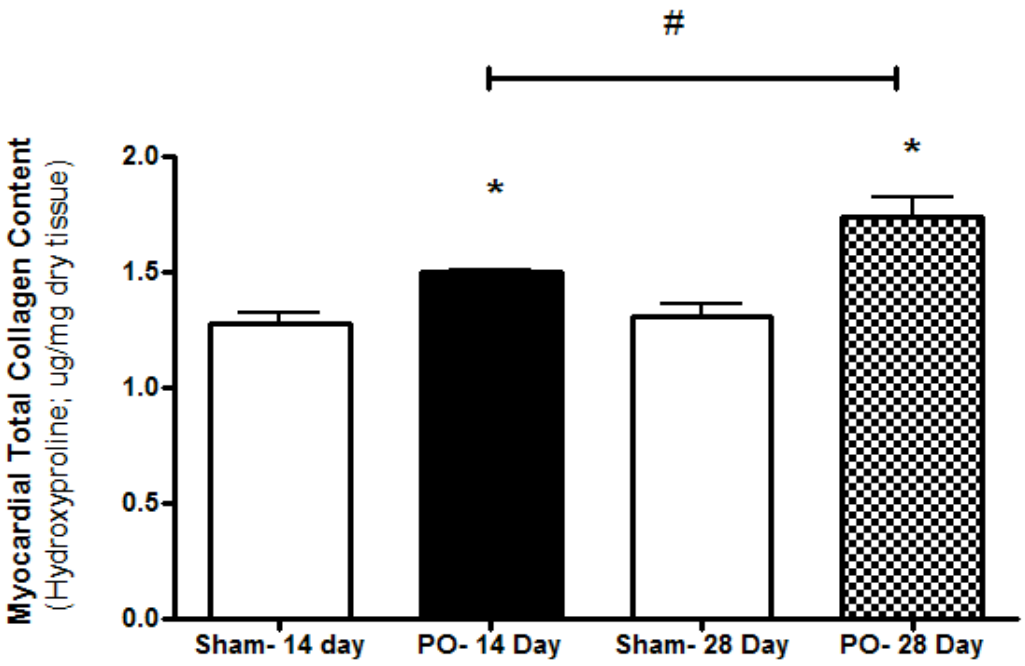


Figure 5: Total collagen measured by hydroxyproline analysis in LV tissue of age-matched sham and 14 and 28 day PO animals. Values are represented as mean ± SEM where *p ≤ 0.05 as compared to the age-matched shams, #p<0.01 as compared to 14 day PO.

Total collagen content in a portion of the left ventricular septal wall was determined by the hydroxyproline concentration. Total collagen content was significantly increased in the 14 day PO animals as compared to their age matched shams as well as the 28 day PO animals as compared to their age matched shams (figure 5). The collagen content in the PO group at 28 days was significantly higher than that in the 14 day PO group, indicating progressive fibrosis. There was no significant difference between either age-matched sham groups (figure 5).

Figure 6: Expression of CB1 receptors on rat cardiac fibroblasts in left ventricular cross-section.

(A)

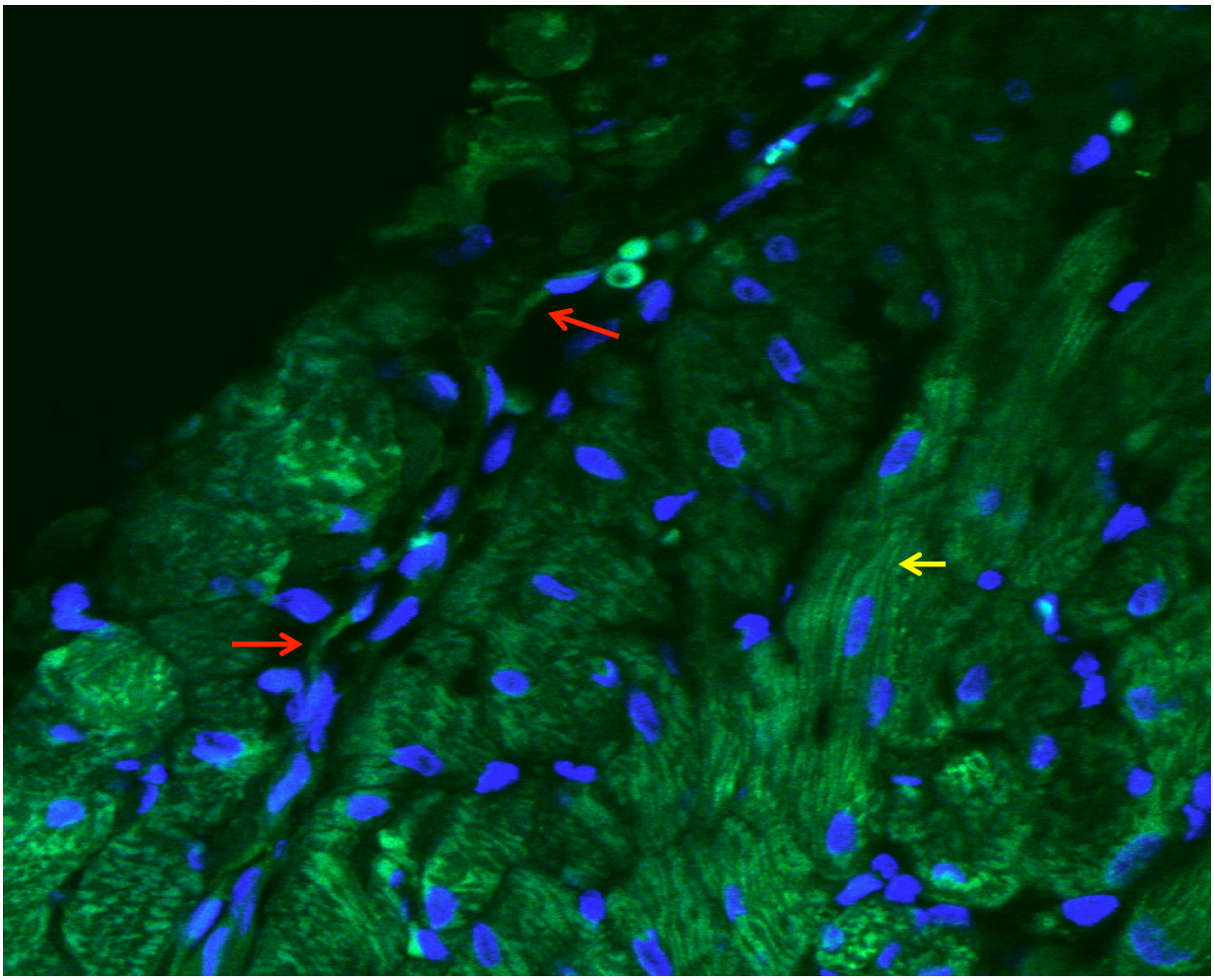


Figure 6(A): Expression of CB1 receptors on rat LV fibroblasts and myocytes. CB1 receptors were detected by using rabbit anti-CB1 antibody and Alexafluor 488 labeled donkey anti-rabbit secondary antibody. Nuclei were counterstained with DAPI. Red arrows point towards fibroblasts. Yellow arrow points towards myocyte.

(B)

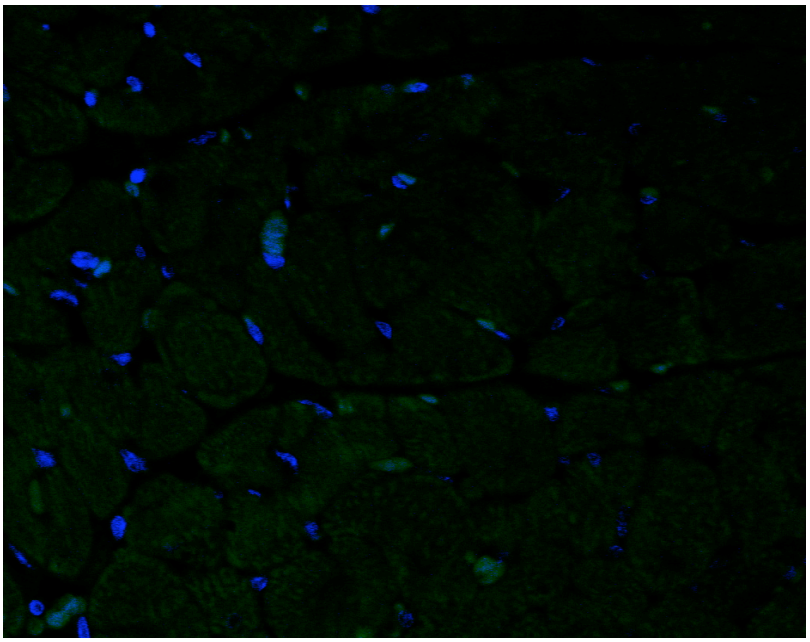


Figure 6B: Negative control for CB1 receptors where anti-CB1 primary antibody was omitted and only Alexafluor 488 labeled secondary antibody was used. Nuclei were counterstained with DAPI.

Expression of CB1 receptors on fibroblasts in rat LV cross-sections was determined by fluorescence microscopy using a rabbit polyclonal anti-CB1 primary antibody and Alexafluor 488 labeled donkey anti-rabbit secondary antibody. Figure 6A shows an apparent expression of CB1 receptors on the fibroblasts and myocytes in the LV section. The nuclei were counterstained blue using DAPI. The absence of non-specific binding of the secondary antibody was confirmed by obtaining a negative control without the use of the anti-CB1 primary antibody and using only the fluorescent secondary antibody (figure 6B).

Figure 7: Temporal expression pattern of myocardial CB1 receptors at 3, 5 day time points in rat pressure overload (PO) model.

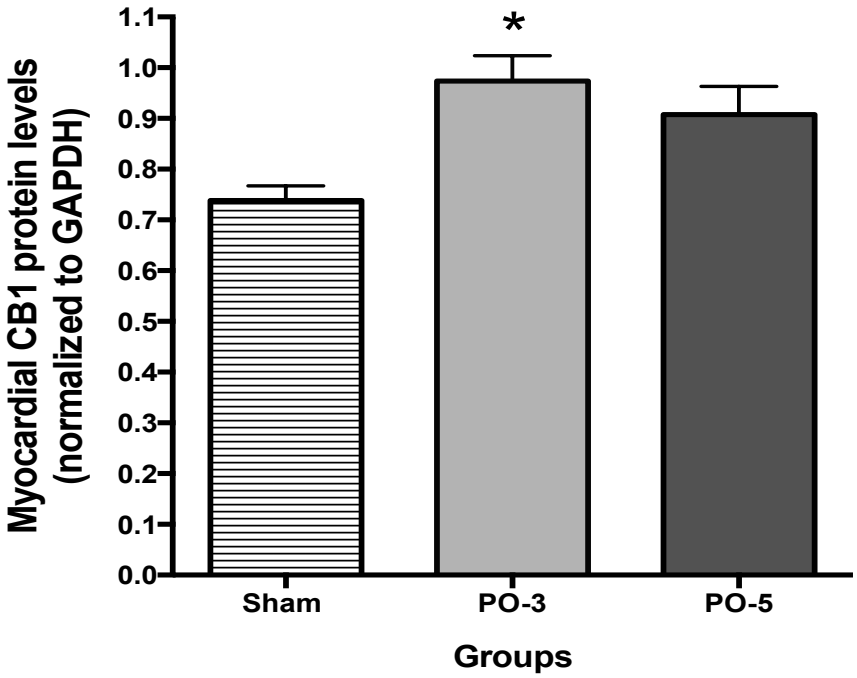


Figure 7: Average Western blot signal intensity of myocardial CB1 receptor protein levels after normalization to GAPDH at 3 and 5 day time points in sham and PO rats. Data are represented as mean ± SEM, n=6/group where *p ≤ 0.05 as compared to sham controls.

Myocardial CB1 receptor expression was measured by western blotting in the 3 and 5 day PO animals and the values were normalized to GAPDH. The CB1 expression was increased significantly at the 3 day time point in PO animals as compared to age-matched sham animals as shown in figure 7. There was a downward trend in expression after day 3 and CB1 expression at 5 days in the PO group was unchanged from age-matched sham values (figure 7).

Figure 8: Temporal expression pattern of myocardial CB1 receptors at 14 and 28 day time points in rat pressure overload model.

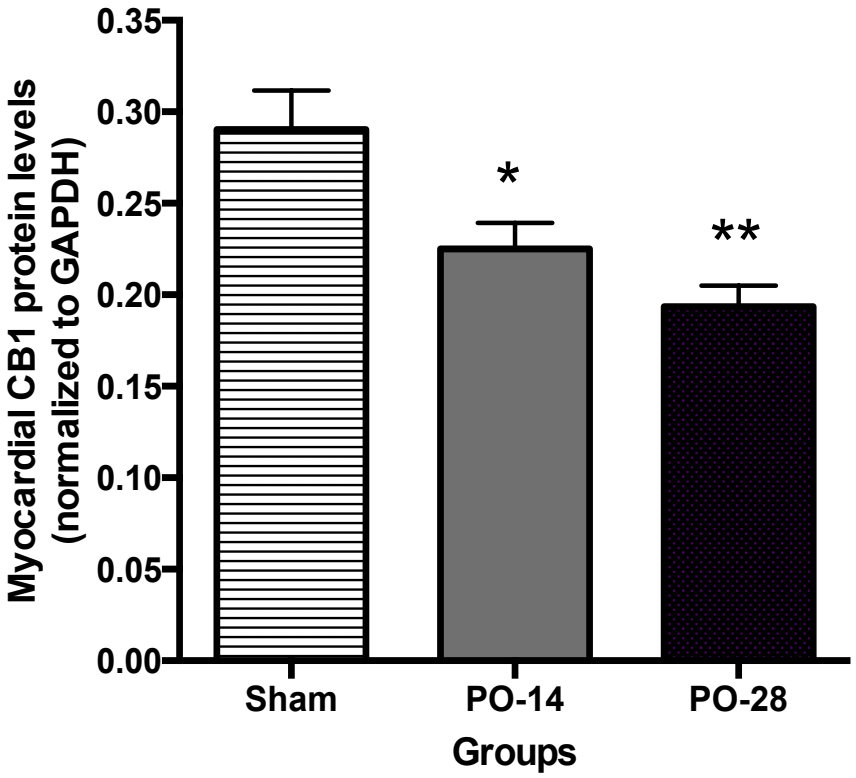


Figure 8: Average Western blot signal intensity of myocardial CB1 receptor protein levels after normalization to GAPDH at 14 and 28 day time points in sham and PO (pressure overload) rats. Data are represented as mean ± SEM, n=6 rats per group where *p ≤ 0.05, **p ≤ 0.01 as compared to sham controls.

Myocardial CB1 receptor expression was measured by western blotting in the 14 and 28 day PO animals. As shown in figure 8, myocardial CB1 receptor levels were significantly decreased at the 14 day time point in the PO group as compared to age-matched sham values. The CB1 expression was also found to be decreased at the 28 day time point in the untreated PO group as compared to sham values (figure 8).

Figure 9: Expression of CB2 receptors on rat cardiac fibroblasts in left ventricular cross-section.

(A)

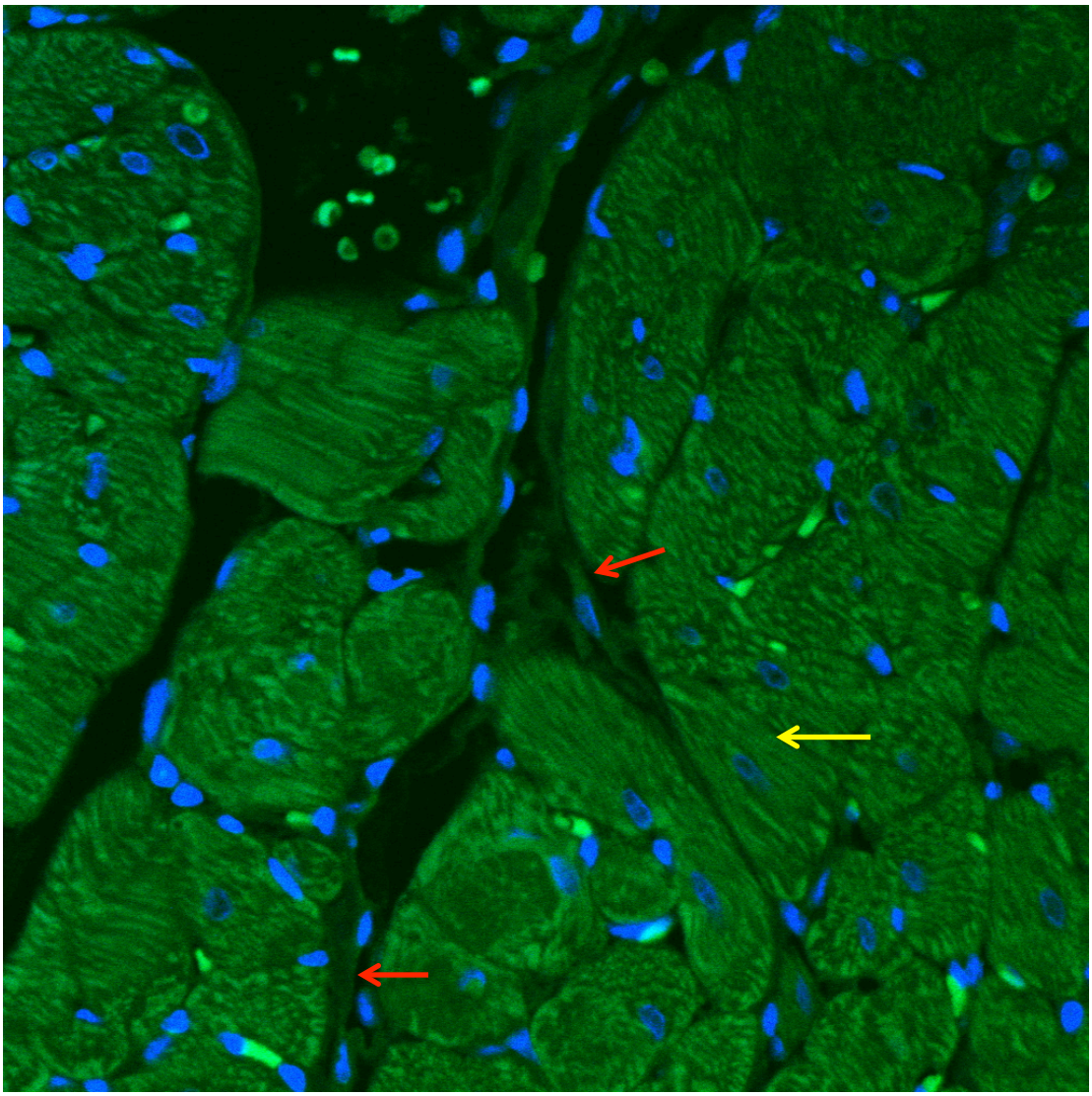


Figure 9(A): Expression of CB2 receptors on rat LV fibroblasts and myocytes. CB2 receptors were detected by using rabbit anti-CB2 antibody and Alexafluor 488 labeled donkey anti-rabbit secondary antibody. Nuclei were counterstained with DAPI. Red arrows point towards fibroblasts. Yellow arrow points towards myocyte.

(B)

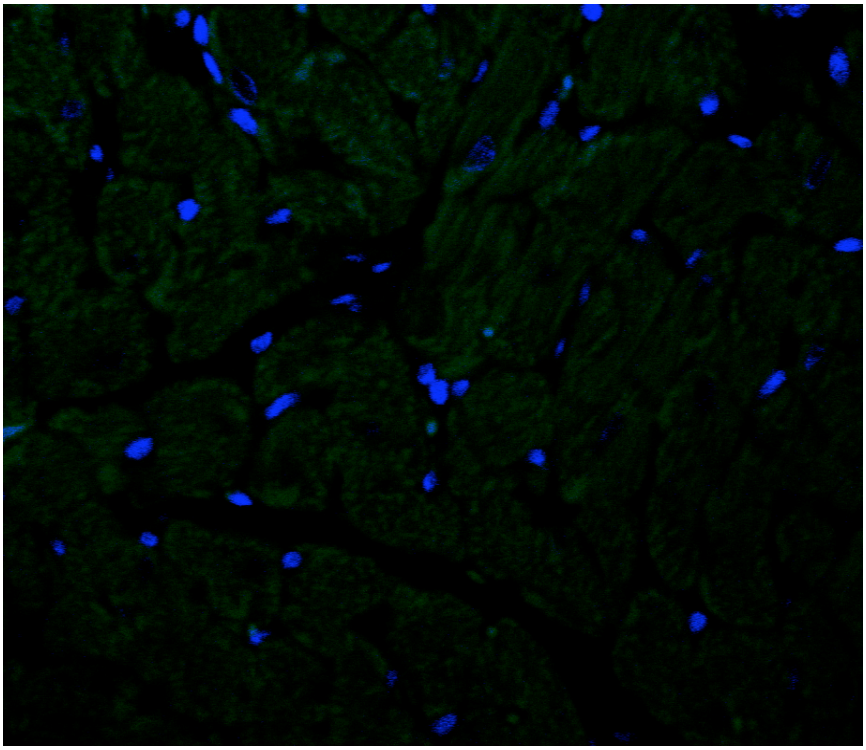


Figure 9B: Negative control for CB2 receptors where anti-CB2 primary antibody was omitted and only Alexafluor 488 labeled secondary antibody was used. Nuclei were counterstained with DAPI.

Expression of CB2 receptors on fibroblasts in rat LV cross-sections was determined by fluorescence microscopy using a rabbit polyclonal anti-CB2 primary antibody and Alexafluor 488 labeled donkey anti-rabbit secondary antibody. Figure 9A shows an apparent expression of CB2 receptors on the fibroblasts and myocytes in the LV section. The nuclei were counterstained blue using DAPI. A negative control was obtained using only the fluorescent secondary antibody and omission of the anti-CB2 primary antibody to ensure the absence of non-specific binding of the secondary antibody (figure 9B).

Figure 10: Temporal expression pattern of myocardial CB2 receptors at 3, 5 day time points in rat pressure overload model.

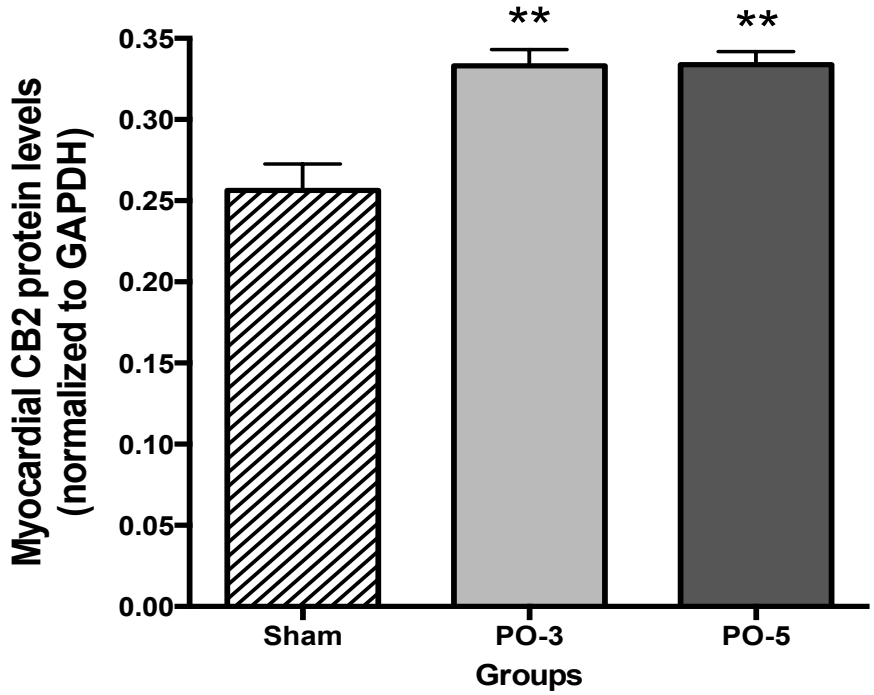


Figure 10: Average Western blot signal intensity of myocardial CB2 receptor protein levels after normalization to GAPDH at 3 and 5 day time points in age-matched sham and PO (pressure overload) rats. Data are represented as mean ± SEM, n=6 mice per group where *p ≤ 0.05, **p ≤ 0.01 as compared to sham control.

Myocardial CB2 receptor expression was measured by western blotting in the 3 and 5 day PO animals and the values were normalized to GAPDH. CB2 receptor expression was significantly increased at both, the 3 and 5 day time points in the untreated PO groups as compared to age-matched sham values (figure 10).

Figure 11: Temporal expression pattern of myocardial CB2 receptors at 14 and 28 day time points in rat pressure overload model

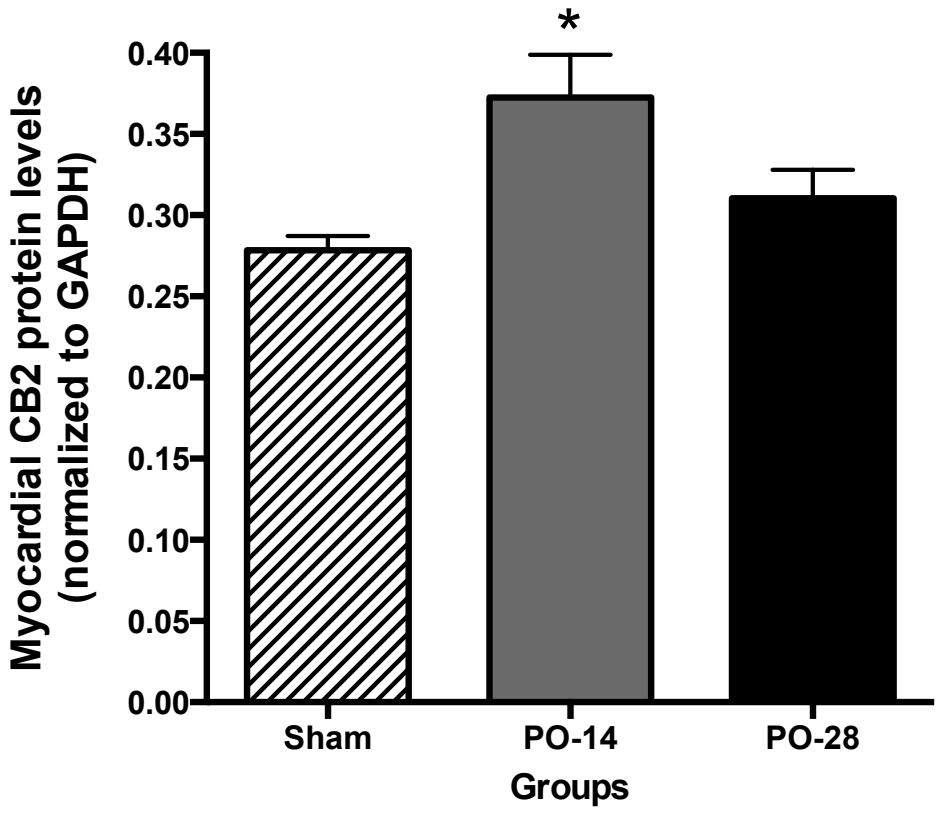


Figure 11: Average Western blot signal intensity of myocardial CB2 receptor protein levels after normalization to GAPDH at 14 and 28 day time points in age-matched sham and PO rats. Data are represented as mean ± SEM, n=6 mice per group where *p ≤ 0.05 as compared to sham control.

Myocardial CB2 receptor expression was measured by western blotting in the 14 and 28 day PO animals and the values were normalized to GAPDH. As shown in figure 11, myocardial CB2 receptor expression was significantly increased only at 14 day time

point in the untreated PO groups as compared to sham values. The receptor expression levels were not significantly different as compared to the age-matched sham group in the 28 day post pressure overload group (figure 11).

DISCUSSION

Hypertension is a common disorder affecting a very large population, with the numbers increasing every year and the recent statistics showing about 30% incidence among the US population (Go et al., 2013). Prolonged and severe hypertension leads to cardiac remodeling and an increased risk of developing heart failure. Current therapies are only partially beneficial at preventing or reversing the adverse remodeling changes affecting cardiac function (Gustafsson et al., 2010). Thus, there is a need for improved therapies for preventing or attenuating the pathophysiologic changes in remodeling. The endocannabinoid system has been recently implicated in mediating protective effects in tissue remodeling associated with various cardiovascular disorders. However, there is a limited knowledge base exploring the role of the ECS in cardiac remodeling secondary to hypertension.

In the present study, we determined the expression of CB1 and CB2 receptors on cardiac fibroblasts in the rat LV cross-sections. These receptors have been discovered in the cardiovascular system in the recent years on human cardiomyocytes (Bonz et al., 2003; Weis et al., 2010) and human endothelial and smooth muscle cells (Liu et al., 2000; Rajesh et al., 2007, 2008a) but they have not been previously demonstrated directly on cardiac left ventricular fibroblasts. Our immunohistochemistry results indicate that both these receptors are expressed on resident cardiac fibroblasts and also on

myocytes in the rat LV sections. CB1 expression on rat fibroblasts in the LV has been demonstrated for the first time while expression on myocytes has been previously reported (Mukhopadhyay et al., 2010). CB2 expression on rat fibroblasts in the LV has also been demonstrated for the first time while expression on myocytes has been previously reported (Lepicier et al., 2003).

Previous work has not elucidated whether the myocardial expression of CB1 and CB2 receptors is altered in a temporal manner as the heart undergoes hypertrophic and fibrotic changes with progressive remodeling secondary to pressure overload or remodeling induced by hypertension. To elucidate this, we induced pressure overload by abdominal aortic constriction (AAC) in four groups of rats (3, 5, 14 and 28 day PO) and included groups of age-matched controls also known as sham animals (surgical procedure without constriction of aorta). The animals were rendered hypertensive by AAC as evidenced by an increase in the mean arterial pressure, which was significantly higher in the 14 and 28 day PO groups as compared to their age matched controls. Hypertrophy was apparent by significantly increased LV mass and LV/body weight index at the 14 and 28 day time points in the PO groups. Cardiac fibroblasts cause fibrosis by producing and depositing excessive collagen in the extracellular matrix and fibrosis is also a major part of cardiac remodeling as previously described above. Incidence of fibrosis was determined by measuring the total collagen content in the LV sections by hydroxyproline analyses. Fibrosis was evident at the 14 day time point in the PO group and also increased in the 28 day group as compared to their age matched controls. The fibrosis was found to be progressive since the elevation in total collagen content at the 28 day time point was significantly higher as compared to that at the 14 day time point.

In a stressed myocardium, fibroblasts secrete the chemokine, monocyte chemoattractant protein-1 (MCP-1) (Behr et al., 2000; Manabe et al., 2002), which stimulates the production of profibrotic cytokine and growth factors that stimulate the fibroblasts in an autocrine and paracrine manner to produce collagen. Under pathological stimuli, MCP-1 expression is also induced in the endothelial cells, which can cause immune cell infiltration and release of proinflammatory and profibrotic factors such as TNF-α, TGF-β and PDGF, which stimulate hypertrophic and fibrotic responses (Dobaczewski and Frangogiannis, 2010). The AAC induced pressure overload model causes extensive remodeling of myocytes and the ECM. However, it only shows an initial transient inflammatory response and does not lead to oxidative stress, a prolonged infiltration of inflammatory cells and loss of myocytes such as that seen in models of ischemia and infarction. A study by Higashiyama et al. (2007) demonstrated an intense initial infiltration of macrophages and neutrophils on day 3 after induction of pressure overload, which had declined thereafter by day 10. Our study showed an elevation in MCP-1 levels at day 3 post induction of pressure overload in the initial inflammatory phase and a decline thereafter by day 5. This effect was similar to the previous study by Kuwahara et al. (2004) showing peak MCP-1 mRNA expression at day 3 post induction of pressure overload in rats with a subsequent decline after day 3. Thus, in agreement with previous work, MCP-1 was induced in the initial inflammatory phase for recruitment of macrophages and neutrophils in the myocardium.

Studies have implicated the endocannabinoid system in cardiac remodeling associated with ischemia and cardiomyopathy, with CB1 receptor antagonists and CB2 receptor agonists linked to beneficial effects. We measured the myocardial CB1 and

CB2 receptor temporal expression at 3, 5, 14 and 28 day time points post induction of pressure overload. CB1 receptor expression was increased significantly at the 3 day time point in PO animals after which there was a downward trend in expression and the expression at 5 days in the PO group was unchanged from that in age-matched sham animals. The downward trend of CB1 expression continued further and CB1 receptor levels were significantly decreased at 14 and 28 days in the PO groups as compared to age-matched sham values. Myocardial CB2 protein level was significantly increased at the 3, 5 and 14 day time points in the untreated PO groups as compared to age-matched sham values. Subsequently, myocardial CB2 receptor expression was similar to age-matched sham levels at the 28 day time point. Thus, myocardial CB1 and CB2 receptors seem to be temporally altered in cardiac remodeling induced by pressure overload.

Previous studies have shown CB1 receptor antagonism to be protective against various cardiovascular disease states (Mukhopadhyay et al., 2007; Rajesh et al., 2008; Dol-Gleizes et al., 2009, Slavic et al., 2013). CB1 antagonists were shown to have a protective effect against myocardial infarction with reduction in infarct size on treatment with rimonabant, a synthetic CB1 antagonist (Lim et al., 2009). Mukhopadhyay and group (2010) have demonstrated reduced levels of collagen and profibrotic factors in CB1 null mice in a model of doxorubicin induced oxidative stress and fibrosis. CB1 antagonism and CB1 knockout animal models have also been shown to exert beneficial effects in hepatic fibrosis (Chen et al., 2012; Giannon et al., 2012). Contradictory findings also exist showing adverse effects of CB1 antagonism (Wagner et al., 2003; Mendizabal and Graschinsky, 2007). A recent study by Liao et al., 2012 showed

deletion of CB1 receptors promoting cardiac remodeling via activation of epidermal growth factor receptor and MAP kinases. More studies are needed to delineate the full effects of modulating the CB1 receptors in cardiovascular pathology, though majority of the studies tend towards blockade of CB1 receptors showing beneficial effects. We can speculate that the temporal decrease in CB1 receptor expression at the 14 and 28 day time points found in our study could be in agreement with the protective effects of CB1 antagonism and could be a physiological response of the remodeling heart to limit the adverse pathological changes. The transient increase in CB1 expression at 3 days before it began the downward trend could be attributed to the initial inflammatory phase after induction of pressure overload seen in our model. Studies have shown that CB1 receptors are expressed on immune cells, although to a lesser extent than CB2 receptors, and they play a role in immune cell modulation (Kaplan, 2013).

CB2 receptor activation has been shown to mediate cardioprotective effects in ischemia and reduction of infarct size (Lepicier et al., 2003). A study by Di Fillipo et al. (2004) with WIN-55212-2 (non-specific CB1 and CB2 agonist) showed a reduction in reperfusion injury and infarct size as well as decreased activation of macrophages and local generation of cytokines. Further, this beneficial effect was blocked by a specific CB2 antagonist (Di Fillipo et al., 2004). CB2 receptor activation has also demonstrated beneficial effects in counteracting hepatic fibrosis (Julien et al., 2005; Munoz-Luque et al., 2008). Evidence shows CB2 receptors can limit post-ischemic fibrosis (Defer et al., 2009). In our current study, CB2 receptor expression could be increased on the fibroblasts at the 3, 5 and 14 day time points to limit the extent of fibrosis. Another possibility to consider is that the increase in expression seen at 3 and 5 days could be

contributed by the initial influx of immune cells in our model as CB2 receptors are known to be widely expressed on different types of immune cells (Miller and Stella, 2008).

CONCLUSION

The present study shows that CB1 and CB2 receptors are expressed on cardiac fibroblasts as demonstrated in rat LV cross-sections. The expression of these receptors is also seen on rat cardiac myocytes in agreement with previous work showing their existence on myocytes (Bonz et al., 2003; Lepicier et al., 2003). The CB1 receptor expression is altered temporally in the PO animals with the expression transiently going up at 3 days and then significantly decreasing at the 14 and 28 day time points. The CB2 receptor expression is also altered temporally in the PO animals with the expression markedly increasing at the initial 3 and 5 day time points, possibly in response to the initial inflammatory response in the PO model. CB2 receptor expression is also found to be up at the 14 day time point in the PO animals while returning to control levels by day 28. The temporal alterations in CB1 and CB2 receptors in cardiac remodeling are an interesting finding, which demonstrates that the ECS is responsive to pathophysiological changes associated with pressure overload induced cardiac remodeling.

AIM 2

Aim 2: To determine the effects of CB1 cannabinoid receptor modulation on human ventricular fibroblasts (HVF).

Aim 2.1: To determine expression of CB1 receptors on HVF

Aim 2.2: To measure changes in total collagen and expression of collagen type 1A and type 3A after treatment of CB1 antagonist, AM251.

Aim 2.3: To determine levels of MCP-1 after treatment of CB1 antagonist, AM251

Rationale:

Studies have established that blockade of CB1 receptors can prevent fibrosis in the heart in doxorubicin induced cardiomyopathy (Mukhopadhyay et al., 2010; Slavic et al., 2013) and in the liver (Patsenkar et al., 2011; Giannon et al., 2012). CFs are the primary cells involved in fibrosis and ECM remodeling. Although CFs are stimulated by numerous neurohormonal chemicals, vasoactive peptides and cytokines which affect their downstream activities promoting fibrosis, the role of the endocannabinoid system in promoting this deleterious remodeling is uncertain (Weber et al., 1994; Segura et al. 2012). Expression of CB1 receptors have been shown in various cell types of the heart (Bonz et al., 2003; Rajesh et al., 2008; Liu et al., 2000), but to date, expression of these

receptors on human ventricular fibroblasts (HVF) has not been documented and the effects of modulating the activities of CB1 receptors directly on the HVF remain to be elucidated. We hypothesize that CB1 receptors are expressed on human ventricular fibroblasts and that antagonism of the CB1 receptors would have an antifibrotic effect.

Experimental Approach:

Human ventricular fibroblasts (HVF) were grown to 80% confluence in 100 mm cell culture plates in DMEM with 10% FBS and 5% NCS. Cells were serum starved for 24 hours with DMEM containing 2% FBS before treatment with 2-arachidonoyl glycerol (2-AG) (endocannabinoid agonist), the synthetic CB1 antagonist, AM251, or a combination of 2-AG+AM251. These treatments were carried out for 48 hours with re-dosing after 24 hours. Fibroblasts were stimulated with 2-AG, 30 minutes after treatment with AM251 in the combined treatment group. Each treatment was performed in triplicate in two independent studies. The culture media was collected from all the plates at the end of the treatment for determination of total secreted collagen and MCP-1. Cell lysates were obtained for protein quantification. For qPCR studies, HVF were grown to 80% confluence in 60 mm cell culture dishes in DMEM with 10% FBS and 5% NCS. Cells were serum starved for 24 hours with DMEM containing 2% FBS before treatment with 2-AG and AM251. Each treatment was performed in triplicate in two independent experiments. Cell lysates were prepared, RNA was extracted using the RNeasy Mini kit (Qiagen, Valencia, CA) and qPCR was performed on each RNA sample in duplicate in two independent experiments for measuring mRNA levels of collagen 1A and 3A. FAM labeled TaqMan collagen 1A1 (ref no. Hs00164004_m1) and collagen

3A1 (ref no. Hs00943809_m1) primers and VIC labeled TaqMan GAPDH primer (ref no. Hs02758991_g1) from Applied Biosystems (Grand Island, NY) were used for qPCR studies. The different groups and drug concentrations for all of the above experiments are outlined below in table 3. Immunofluorescence was performed on HVF fixed on 8-chamber slides for determining the expression of CB1 receptors using a rabbit polyclonal anti-CB1 primary antibody (1:100 in 1% BSA in PBS) (Thermo Scientific, Rockford, IL) and Alexafluor 488 labeled goat anti-rabbit secondary antibody (1:500 in 1% BSA in PBS) (Molecular Probes, Grand Island, NY).

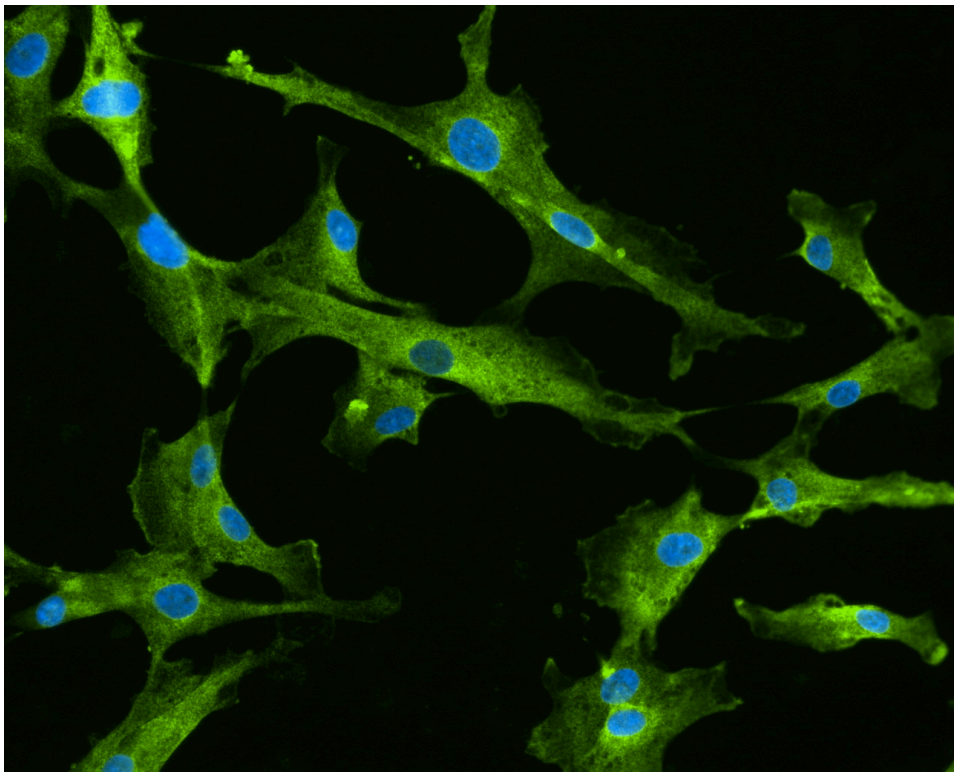
Table 3: Treatment groups for CB1 antagonism studies on HVF

Drugs	Doses
Control	Untreated
Vehicle (Ethanol)	10 uM
2- Arachidonoyl glycerol (2-AG)	10 uM
AM251	10 uM
2-AG + AM251	10 uM + 10 uM

RESULTS

Figure 12: Expression of CB1 receptors on human ventricular fibroblasts.

(A)



(B)

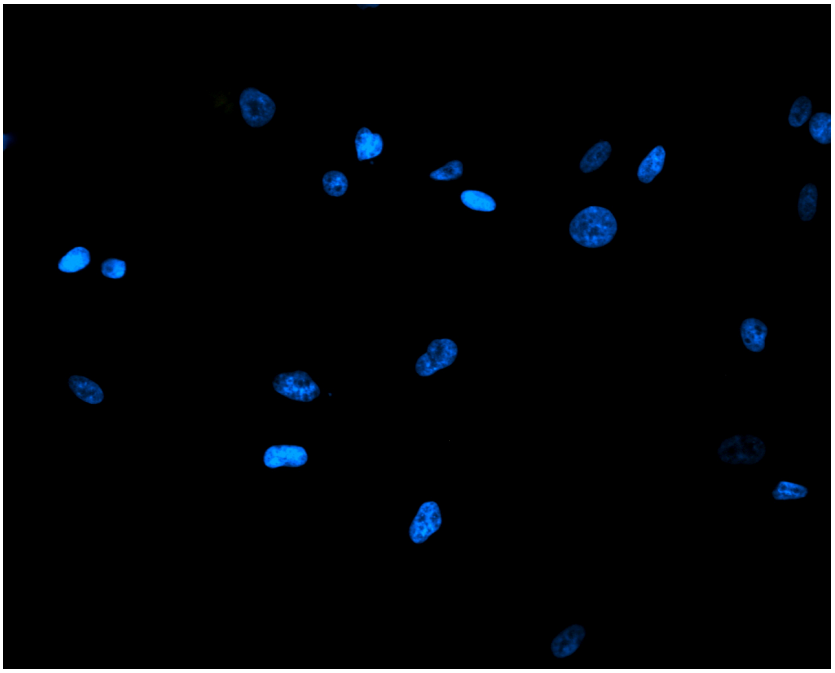


Figure 12: (A) Expression of CB1 receptors on human ventricular fibroblasts. CB1 receptors were detected by using rabbit anti-CB1 antibody and Alexafluor 488 labeled goat anti-rabbit secondary antibody. Nuclei were counterstained with DAPI. (B) Negative control for CB1 receptors where anti-CB1 primary antibody was omitted and only Alexafluor 488 labeled secondary antibody was used. Nuclei were counterstained with DAPI.

Expression of CB1 receptors on human ventricular fibroblasts was determined by fluorescence microscopy using a rabbit polyclonal anti-CB1 primary antibody and Alexafluor 488 labeled goat anti-rabbit secondary antibody. As seen in figure 12(A), CB1 receptors were apparently expressed on the membranes of the human ventricular fibroblasts. The nuclei were counterstained blue using DAPI (4,6- diamidino-2-phenylindole). The absence of non-specific binding of the secondary antibody was confirmed by obtaining a negative control without the use of the anti-CB1 primary antibody and using only the fluorescent secondary antibody [figure 12(B)].

Figure 13: Detection of CB1 receptors on human ventricular fibroblasts by Western Blotting

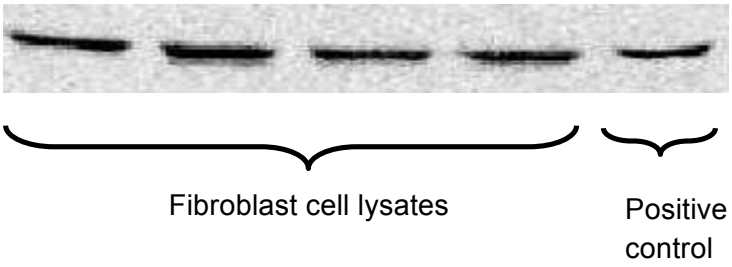


Figure 13: Representative western blot showing expression of CB1 receptors on human ventricular fibroblasts. Cell lysates constituting 40 ug of protein/well were loaded and analyzed using rabbit polyclonal anti-CB1 antibody. Positive control used was mouse hippocampus.

The presence of CB1 receptors on human ventricular fibroblasts was also determined by detection with the anti-CB1 primary antibody using western blots as shown in figure 13. Each lane was loaded with 40 ug of protein using fibroblast cell extracts. A band specific for the CB1 receptor was detected at approximately 60 kD. The positive control used was mouse hippocampus, which is known to have an abundance of CB1 receptors (Pacher et al., 2006).

Figure 14: Total collagen secreted by fibroblasts is altered by CB1 antagonist

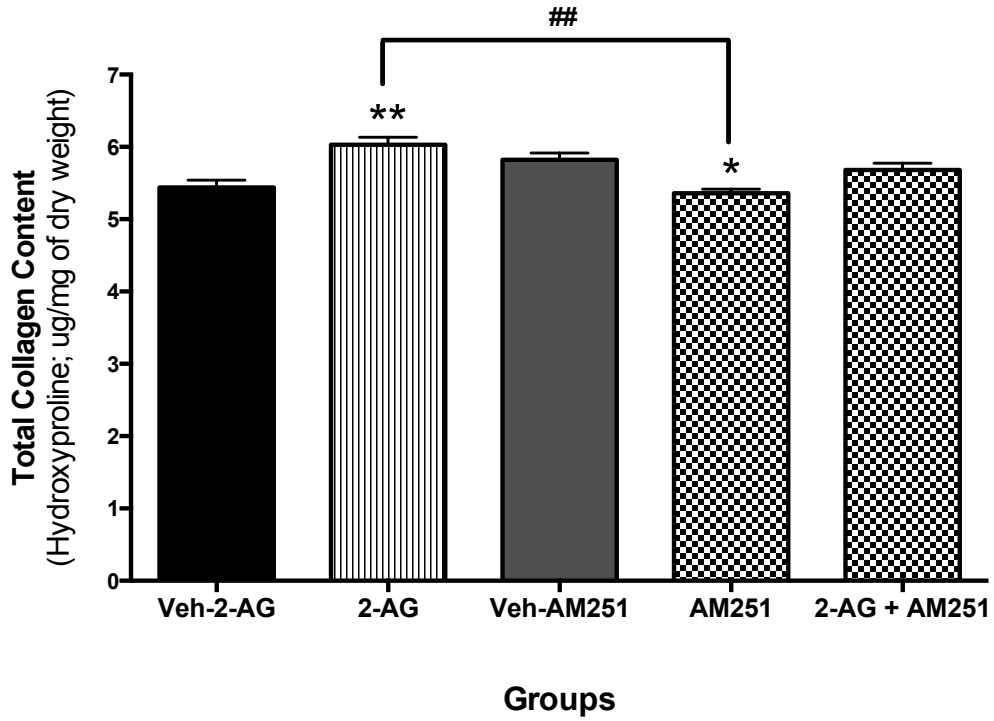
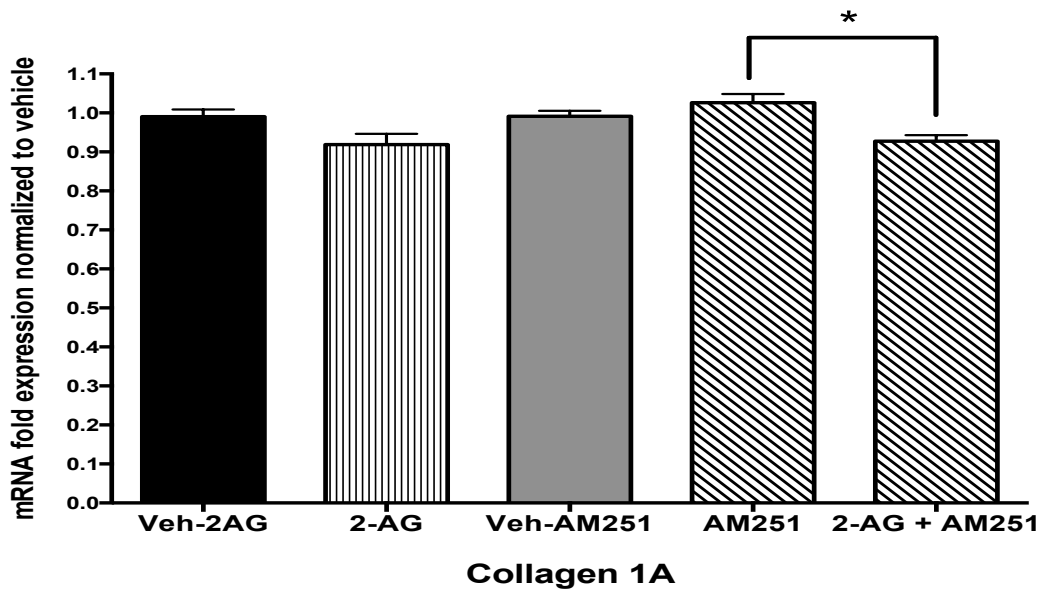


Figure 14: Total collagen secreted in the culture media by human ventricular fibroblasts measured by hydroxyproline analysis on treatment with 2-AG (10uM), AM251 (10uM) and 2-AG + AM251 (both 10uM). Values are represented as mean ± SEM where *p ≤ 0.05, **p ≤ 0.01 as compared to the respective vehicle control and ##p<0.01 as compared to 2-AG.

Total collagen secreted by human ventricular fibroblasts into the culture media was determined by assessing the hydroxyproline concentration. Total collagen secreted was significantly increased by 2-AG treatment as compared to the vehicle control (figure 14). Treatment with the CB1 antagonist, AM251, by itself significantly decreased the total collagen secreted as compared to the vehicle control as well as compared to 2-AG alone. However, this decrease was not seen when fibroblasts were co-treated with 2-AG + AM251 as compared to the vehicle or 2-AG alone (figure 14).

Figure 15: Collagen 1A and 3A expression is responsive to CB1 antagonist

(A)



(B)

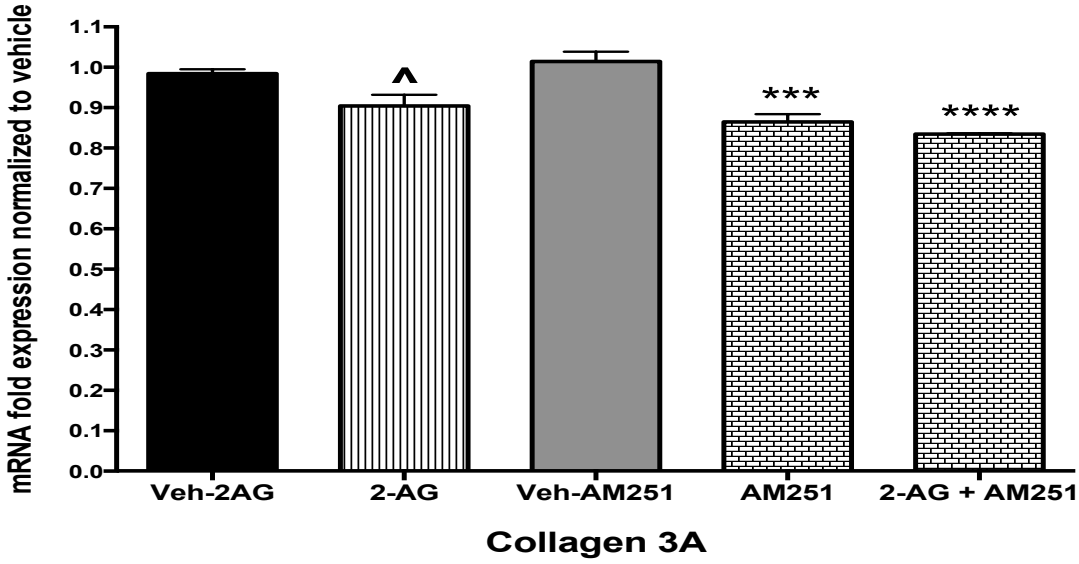


Figure 15: (A) Collagen 1A and (B) collagen 3A mRNA expression in 2-AG (10uM), AM251 (10uM) and 2-AG + AM251 (both 10uM) treated human ventricular fibroblasts normalized to their respective vehicles as measured by qPCR. Values represented as mean ± SEM where ***p ≤ 0.001 as compared to the vehicle control for AM251 and ^ p ≤ 0.05 as compared to vehicle control for 2-AG.

mRNA expression of Collagen 1A and 3A in human ventricular fibroblasts following drug treatments was determined by quantitative real time PCR analyses. There was a significant difference between collagen 1A mRNA expression obtained after treatment with AM251 and 2-AG+AM251 [figure 15(A)]. As seen in figure 15(B), collagen 3A mRNA expression was significantly decreased by 2-AG treatment as compared to the vehicle control. Treatment with the CB1 antagonist, AM251, significantly decreased collagen 3A mRNA expression as compared to the vehicle control. This decrease was also seen when the fibroblasts were co-treated with 2-AG + AM251 as compared to the vehicle control [figure 15(B)].

Figure 16: MCP-1 secreted by fibroblasts is altered by AM251 treatment

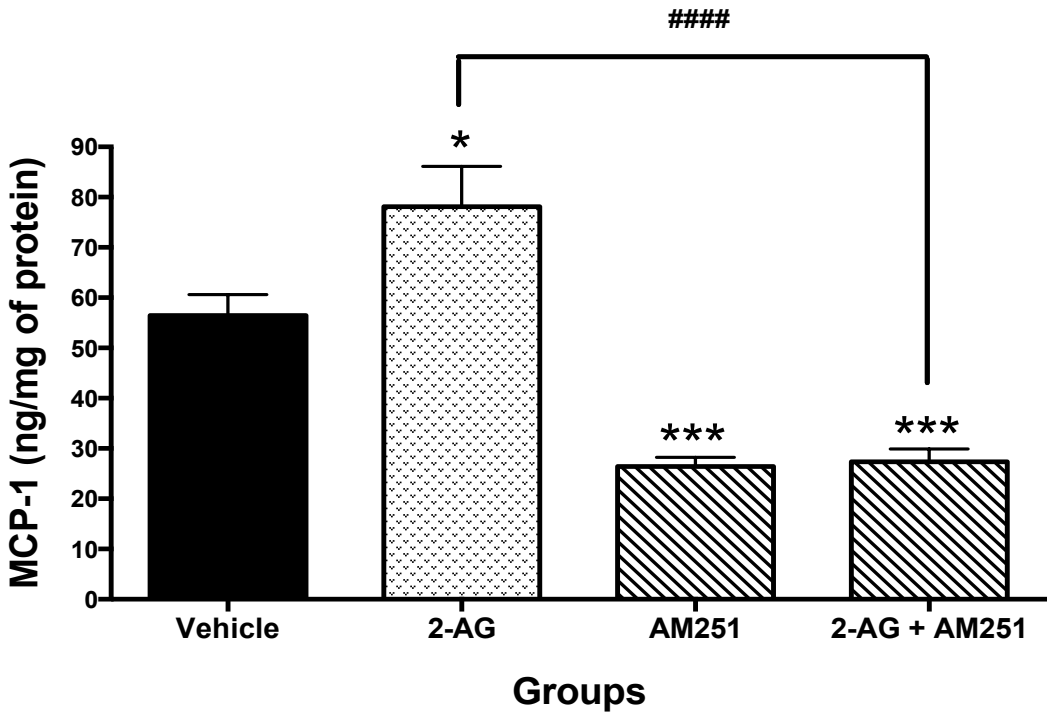


Figure 16: MCP-1 (ng/mg of protein) secreted in the culture media by human ventricular fibroblasts on treatment with 2-AG (10uM), AM251 (10uM) and 2-AG + AM251 (both 10uM) measured by ELISA. Values are represented as mean ± SEM where *p ≤ 0.05, ***p ≤ 0.001 as compared to the vehicle control and ####p<0.001 as compared to 2-AG.

MCP-1 secreted in the culture media by human ventricular fibroblasts was analyzed by a commercially available ELISA kit. MCP-1 secreted in the media was significantly increased by 2-AG as compared to the vehicle control as seen in figure 16. Treatment of fibroblasts with CB1 antagonist, AM251, as well as with co-treatment of 2-AG + AM251 significantly decreased the amount of MCP-1 secreted in the media as compared to the vehicle control. There was also a significant decrease in MCP-1 levels obtained by combined treatment with 2-AG + AM251 as compared to those obtained by treating with 2-AG alone (figure 16).

DISCUSSION

The endocannabinoid system is a ubiquitous system involved in functions of various organs of the body (Pacher et al., 2006). It was found to be active in the cardiovascular system only recently and subsequent research has implicated it in pathophysiological conditions of this system. Studies have shown involvement of the CB1 and CB2 receptors as well as some novel putative cannabinoid receptors in the observed cardiovascular effects (Pacher and Steffens, 2009). Previously, CB1 receptors were found to be mainly abundant in the brain and they have been identified in the heart and vasculature only in the past decade. A few recent studies have shown their existence in the different components of the cardiac system such as in human atrial myocytes (Bonz et al., 2003), human cardiac myocytes (Mukhopadhyay et al., 2010), human coronary artery smooth muscle cells (Rajesh et al., 2008a) and vascular endothelial cells (Liu et al., 2000) and whole myocardial tissue extracts (Wagner et al., 2003). Here, we have shown the presence of CB1 receptors on human ventricular fibroblasts under basal conditions. CB1 receptors were detected by immunofluorescence using rabbit anti-CB1 polyclonal antibody and the binding to these receptors was confirmed when there was no signal reported in the negative control wherein the use of anti-CB1 antibody was omitted. As previously stated above, cardiac fibroblasts are the primary cells of the ECM involved in maintaining homeostasis of the

ECM and collagen remodeling in fibrotic processes. Collagen is the fundamental substance constituting the extracellular matrix in the heart and mainly consists of two major types, collagen 1 and 3, along with several other minor subtypes (Porter et al., 2009). Excessive collagen production and deposition by cardiac fibroblasts under various pathological stimuli such as hypertension, ischemia and myopathy leads to fibrosis in the extracellular matrix, eventually making the ventricular walls stiffer and affecting cardiac function (Segura et al. 2012; Sullivan and Black, 2013). However, studies to date have not detailed the effects of directly modulating the CB1 receptors on human ventricular fibroblasts and effects on collagen production and the chemokine, MCP-1, which stimulates profibrotic factors.

In the present study, we investigated the effects of CB1 antagonism on the total collagen secreted by HVF. Total collagen secreted in the media by the cells was measured by the hydroxyproline content. An increase in total collagen secretion was seen following treatment with the endocannabinoid agonist, 2-AG. The CB1 antagonist, AM251, significantly decreased the total collagen levels as compared to the vehicle control but not when co-treated with 2-AG. Since blocking the CB1 receptors by AM251 caused a decrease in production and secretion of collagen by HVF, CB1 receptors thus seem to be involved in having a profibrotic effect on collagen synthesis. This effect of CB1 antagonism might translate in to a beneficial effect against excessive collagen production and deposition in a model of cardiovascular fibrosis. The observed anti-fibrotic effect of blocking CB1 receptors on HVF is in agreement with a study by Mukhopadhyay and group (2010) that demonstrated reduced levels of collagen and profibrotic factors such as TGF-β and fibronectin in CB1 null mice in a model of

doxorubicin induced oxidative stress and fibrosis. A recent study has also shown CB1 antagonism by rimonabant to be protective against post ischemic fibrosis and remodeling by reducing the accumulation of collagen in a rat model of myocardial ischemia and infarction (Slavic et al., 2013). Moreover, numerous studies have demonstrated anti-fibrotic effects of CB1 receptor antagonism in liver fibrosis by reversal of developed fibrosis (Patsenkar et al., 2011; Giannon et al., 2012) or knockdown of CB1 receptors preventing activation and production of ECM proteins by hepatic stellate cells (HSCs) (Teixeira-Clerc et al., 2006; Chen et al., 2012). Hepatic stellate cells are analogous to cardiac fibroblasts under fibrotic conditions wherein they are involved in ECM remodeling (Moreira, 2007). CB1 receptor blockade is also known to be beneficial in reducing fibrosis in systemic sclerosis (Palumbo-Zerr et al., 2012) and diabetic cardiomyopathy (Rajesh et al., 2012). In our study, the reason for not seeing the decrease in collagen on co-treatment of AM251 with 2-AG is not clear at this point, but it could possibly be due to involvement of receptors other than CB1 stimulated by 2-AG such as the novel cannabinoid receptors or GPR55 (Begg, 2005; Okuno and Yokomizo, 2011).

The effect of CB1 antagonism on the gene expression of collagen 1A and 3A was determined by qPCR. Fibroblasts treated with 2-AG by itself showed significant reduction in collagen 3A mRNA levels and also a significant reduction on treatment with AM251. The expression of collagen 3A was attenuated by AM251 even after stimulation with 2-AG in the combined treatment group. These results suggest that CB1 antagonism is involved in reducing the expression of collagen 3A and this decrease may have contributed to the reduction in total collagen levels seen with AM251. In case

of 2-AG, the total collagen was increased while the collagen 3A expression was decreased and collagen 1A expression was not significantly altered by 2-AG itself. These conflicting results could be due to the fact that collagen mRNA levels were measured at 24 hours while the total collagen protein levels were determined at 48 hours. The decrease in mRNA levels of collagen 3A could be a transient event while collagen 1A may be altered at a later time point, which could contribute to the increased total collagen protein seen at 48 hours. Collagen 1A levels were significantly reduced by stimulation of the cells with 2-AG after blockade of CB1 receptors with AM251 as compared to those obtained with just AM251 treatment. This could indicate a role of CB2 receptors causing a decrease in collagen 1A after stimulation with 2-AG, since 2-AG is an agonist at both CB1 and CB2 receptors.

Alterations in gene expression of collagen 1A and 3A in cardiac fibroblasts by CB1 antagonism have not been studied before. Previous studies on hepatic fibrosis have shown CB1 blockade to downregulate mRNA expression of procollagen 1A in hepatic stellate cells (Patsenkar et al., 2012) and reduced expression of collagen 1A in the liver (Giannone et al., 2012).

MCP-1 is a chemokine stimulating profibrotic factors and can induce collagen production by cardiac fibroblasts (Dobaczewski and Frangogiannis, 2010). We measured MCP-1 secreted in the culture media after treatment of HVF with AM251. MCP-1 was found to be significantly elevated by treatment of 2-AG alone. This increase could possibly have lead to the increased total collagen secretion seen with 2-AG. Treatment with AM251 robustly decreased the amount of MCP-1 secreted by HVF. The decrease in MCP-1 levels was maintained on treatment of AM251 even after HVF were

stimulated by 2-AG in the combined treatment group. Our results conform to previous studies showing decrease in MCP-1 expression by CB1 blockade or knockdown in HSCs or hepatic fibrosis models (Trebicka et al, 2011; Giannone et al., 2012) which was associated with an anti-fibrotic effect. Thus, antagonism of CB1 receptors seems to be anti-fibrotic and the decrease in total collagen and collagen 3A expression could be a downstream effect of the reduced MCP-1 production by AM251. MCP-1 has been shown to directly stimulate TGF-β_1 production by lung fibroblasts, which can affect their collagen production (Gharaee-Kermani et al., 1996). A study by Slavic et al. (2013) showed that CB1 antagonism by rimonabant dose-dependently prevented increase in TGF- β expression induced by IL-1 in cardiac fibroblasts and TGF-b expression was also found to be reduced in CB1 null mice (Mukhopadhyay et al., 2010). Given those previous reports by others, we speculate that CB1 induced reduction in MCP-1 could decrease expression and activation of the profibrotic factor, TGF-β, in HVF to affect their collagen production. TNF-α is another important profibrotic factor involved in promoting collagen production (Duerrschmid et al., 2013). CB1 blockade has been shown to reduce expression of TNF-alpha in hepatic fibrosis (Trebicka et al, 2011; Giannone et al., 2012), which could also contribute to the anti-fibrotic effect seen with HVF.

CONCLUSION

To the best of our knowledge, expression of CB1 receptors was demonstrated for the first time on HVF by immunofluorescence and western blotting. CB1 antagonism reduced total collagen secretion and expression of collagen 3A by HVF. Blockade of CB1 receptors also decreased the amount of MCP-1, secreted in the culture media. Thus, in accordance with the current literature, our results indicate that CB1 receptors are profibrotic and CB1 antagonism could prove beneficial under fibrotic situations.

AIM 3

Aim 3: To determine the effects of CB2 cannabinoid receptor modulation on human ventricular fibroblasts (HVF).

Aim 3.1: To determine expression of CB2 receptors on HVF

Aim 3.2: To measure changes in total collagen and expression of collagen type 1A and type 3A after treatment of CB2 antagonist, AM630.

Aim 3.3: To determine levels of MCP-1 after treatment of CB2 antagonist, AM630

Rationale:

Previous work has established that cannabinoids acting via CB2 receptors can prevent fibrosis in the heart post ischemia (Defer et al.2009) as well as in other tissues such as liver (Munoz-Luque et al., 2008) and pancreas (Michalski et al., 2008). CB2 receptors have been previously shown to be expressed in the heart (Rajesh et al., 2007; Weis et al. 2010). However, to date, expression of CB2 receptors has not been demonstrated on HVF and it is uncertain whether any cardioprotective effects of CB2 receptors against fibrosis are mediated through cardiac fibroblasts. We hypothesize that CB2 receptors are involved in mediating a

beneficial effect against fibrosis and antagonism of the CB2 receptors expressed on HVF would prove to be profibrotic.

Experimental Approach:

Human ventricular fibroblasts (HVF) were grown to 80% confluence in 100 mm cell culture plates in DMEM with 10% FBS and 5% NCS. Cells were serum starved for 24 hours with DMEM containing 2% FBS before treatment with 2-arachidonoyl glycerol (2-AG) (endocannabinoid agonist), synthetic CB2 antagonist, AM630, or a combination of 2-AG+AM630. These treatments were carried out for 48 hours with re-dosing after 24 hours. Fibroblasts were stimulated with 2-AG, 30 minutes after treatment with AM630 in the combined treatment group. Each treatment was performed in triplicate in two independent studies. The culture media was collected from all the plates at the end of the treatment for determination of total collagen and MCP-1. Cell lysates were obtained for protein quantification. For qPCR studies, HVF were grown to 80% confluence in 60 mm cell culture dishes in DMEM with 10% FBS and 5% NCS. Cells were serum starved for 24 hours with DMEM containing 2% FBS before treatment with 2-AG and AM630. Each treatment was performed in triplicate in two independent experiments. Cell lysates were prepared for RNA extraction and qPCR was performed on each RNA sample in duplicate in two independent experiments for measuring mRNA levels of collagen 1A and 3A. FAM labeled TaqMan collagen 1A1 (ref no. Hs00164004_m1) and collagen 3A1 (ref no. Hs00943809_m1) primers and VIC labeled TaqMan GAPDH primer (ref no. Hs02758991_g1) from Applied Biosystems (Grand Island, NY) were used for qPCR studies. The different groups and drug concentrations for all of the above experiments

are outlined below in table 4. Immunofluorescence was performed on HVF fixed on 8-chamber slides for determining the expression of CB2 receptors using a rabbit polyclonal anti-CB2 primary antibody (1:100 in 1% BSA in PBS) (Thermo Scientific, Rockford, IL) and Alexafluor 488 labeled goat anti-rabbit secondary antibody (1:500 in 1% BSA in PBS) (Molecular Probes, Grand Island, NY)

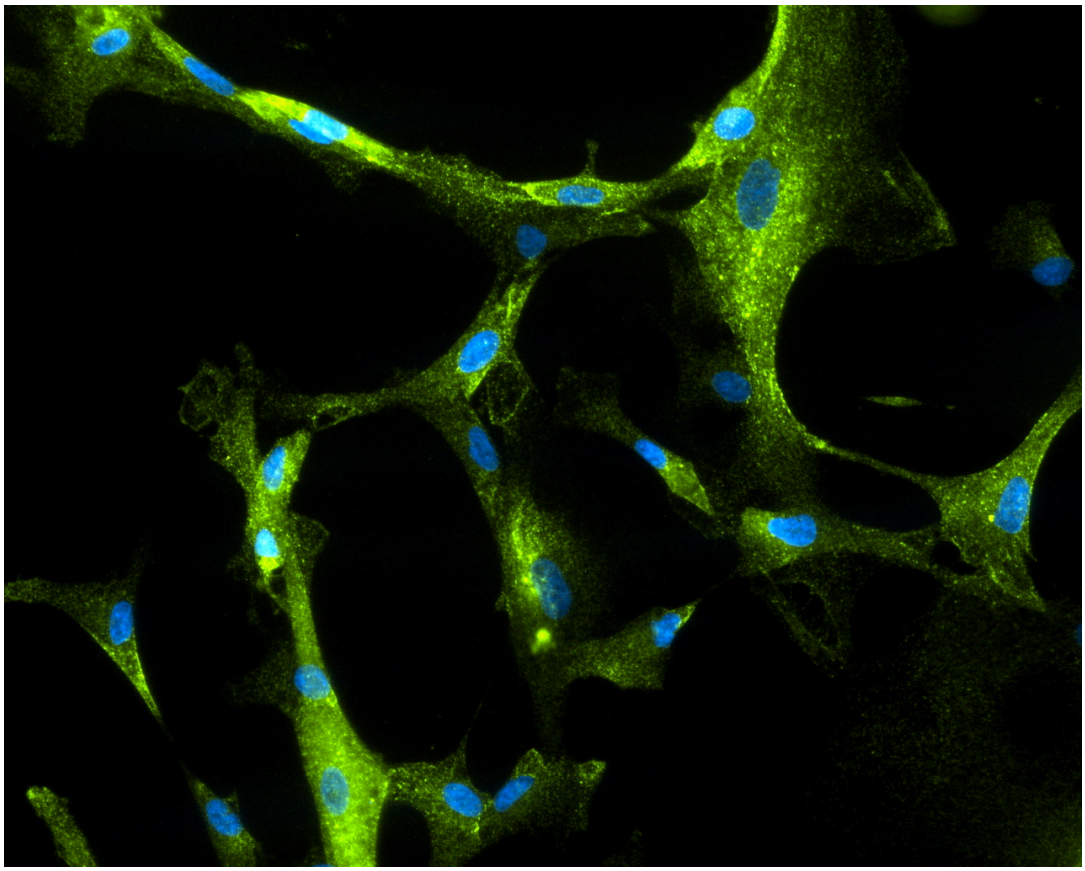
Table 4: Treatment groups for CB2 antagonism studies on HVF

Drugs	Doses
Control	Untreated
Vehicle for 2-AG (Ethanol)	10 uM
Vehicle for AM630 (DMSO)	10 uM
2- Arachidonoyl glycerol (2-AG)	10 uM
AM630	10 uM
2-AG + AM630	10 uM + 10 uM

RESULTS

Figure 17: Expression of CB2 receptors on human ventricular fibroblasts

(A)



(B)

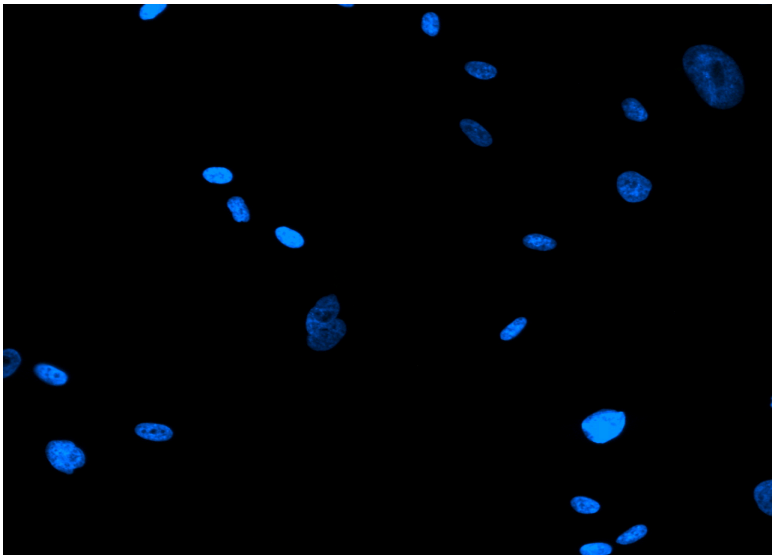


Figure 17: (A) Expression of CB2 receptors on human ventricular fibroblasts. CB2 receptors were detected by using rabbit anti-CB2 antibody and Alexafluor 488 labeled goat anti-rabbit secondary antibody. Nuclei were counterstained with DAPI. (B) Negative control for CB2 receptors where anti-CB2 primary antibody was omitted and only Alexafluor 488 labeled secondary antibody was used. Nuclei were counterstained with DAPI.

Expression of CB2 receptors on human ventricular fibroblasts was determined by fluorescence microscopy using a rabbit polyclonal anti-CB2 primary antibody and Alexafluor 488 labeled goat anti-rabbit secondary antibody. Figure 17(A) show an apparent expression of CB2 receptors on the membranes of the human ventricular fibroblasts. The nuclei were counterstained blue using DAPI. A negative control was obtained using only the fluorescent secondary antibody and omission of the anti-CB2 primary antibody to ensure the absence of non-specific binding of the secondary antibody (figure 17B).

Figure 18: Detection of CB2 receptors on the human ventricular fibroblasts by Western Blotting

Figure 18: Representative western blot showing expression of CB2 receptors on human ventricular fibroblasts. Cell lysates constituting 40 ug of protein/well were loaded and analyzed using rabbit polyclonal anti-CB2 antibody. Positive control used was mouse hippocampus.

The presence of the CB2 receptors on the human ventricular fibroblasts was also determined by detection with the anti-CB2 primary antibody in western blots as shown in figure 18. Each lane was loaded with 40 ug of protein using fibroblast cell extracts. A band specific for the CB2 receptor was detected at approximately 40 kD. The positive control used here was mouse hippocampus, which is known to express CB2 receptors (Pacher et al., 2006)

Figure 19: Total collagen secreted by fibroblasts is altered by CB2 antagonist

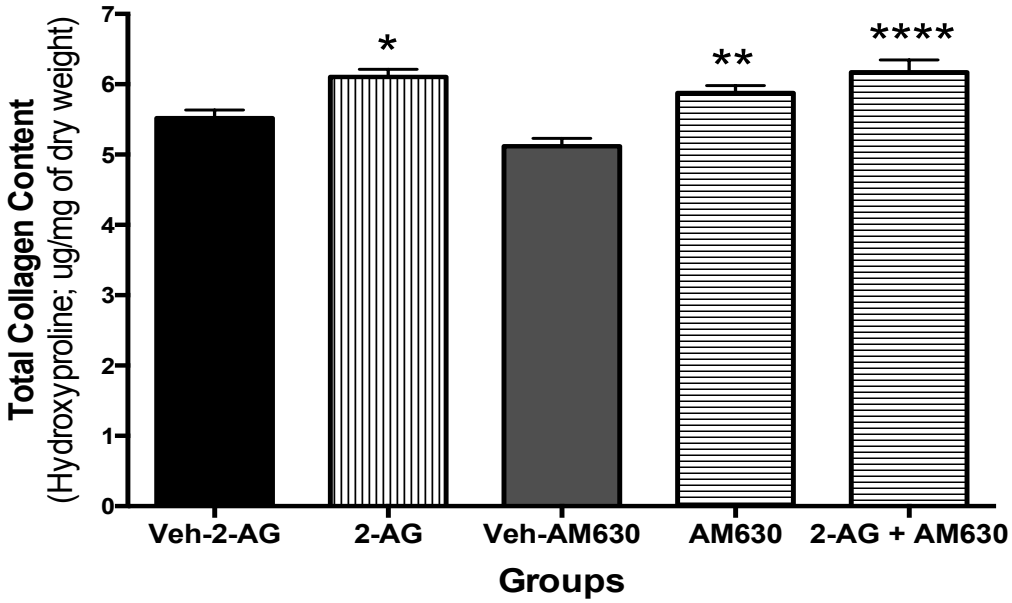


Figure 19: Total collagen secreted in the culture media by human ventricular fibroblasts was measured by hydroxyproline analysis on treatment with 2-AG (10uM), AM630 (10uM) and 2-AG + AM630 (both 10uM). Values are represented as mean ± SEM where *p ≤ 0.05, **p ≤ 0.01 and ****P ≤ 0.001 as compared to the respective vehicle control.

Total collagen secreted by human ventricular fibroblasts in the culture media was determined by the hydroxyproline concentration. As seen in figure 19, total collagen secreted was significantly increased by 2-AG treatment as compared to the vehicle control. Treatment with CB2 antagonist, AM630, significantly increased the total collagen secreted by fibroblasts as compared to the vehicle control. Total collagen secreted in the media was also significantly increased when fibroblasts were co-treated with 2-AG + AM630 and the increase seen was even higher than that with AM630 alone. There was no significant difference between levels of collagen obtained with 2-AG alone and combined treatment of 2-AG + AM630 (figure 19).

Figure 20: Collagen 1A and 3A expression is responsive to CB2 antagonist

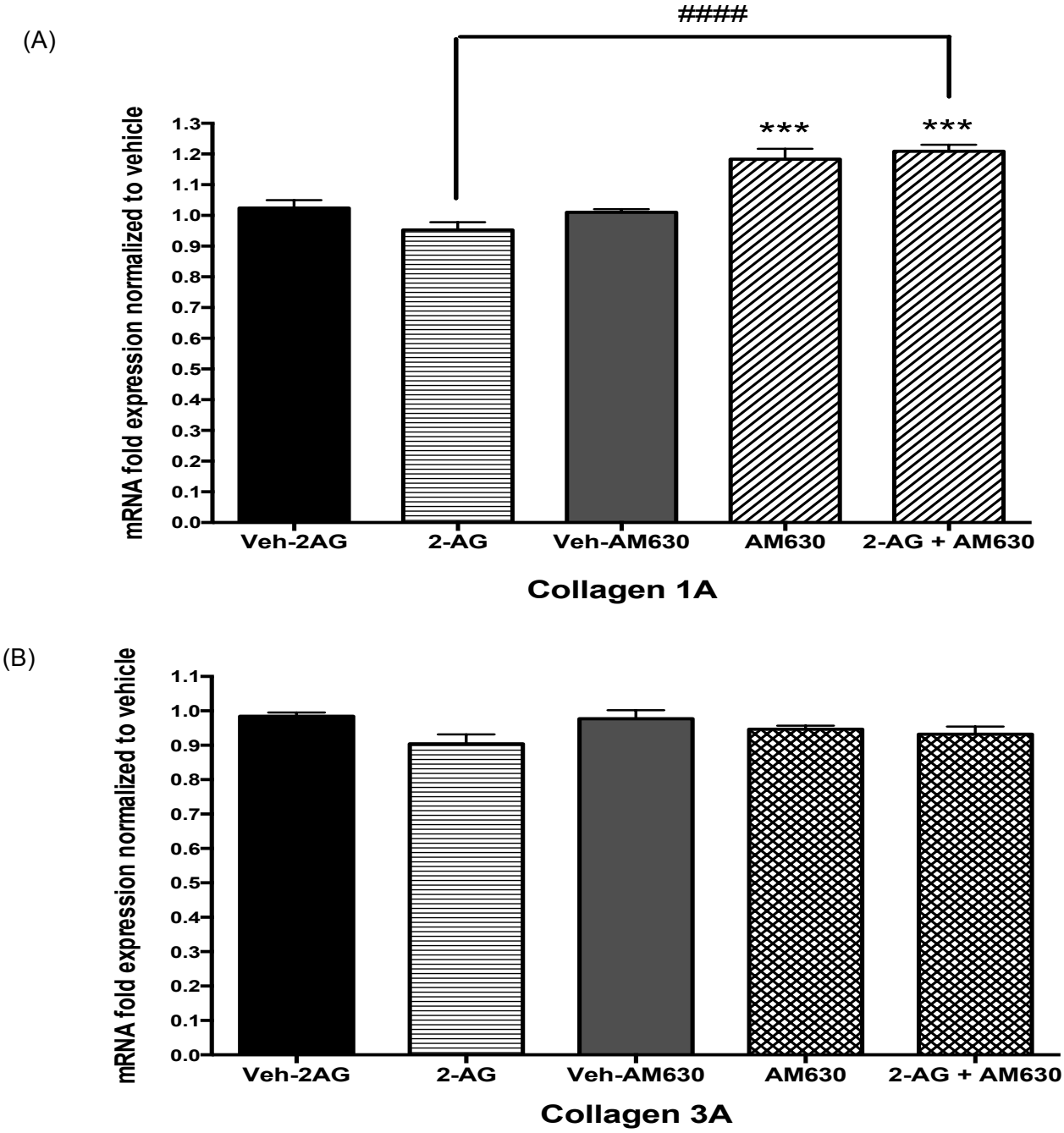


Figure 20: (A) Collagen 1A and (B) collagen 3A mRNA expression in 2-AG (10uM), AM630 (10uM) and 2-AG + AM630 (both 10uM) treated human ventricular fibroblasts normalized to their respective vehicles as measured by quantitative real time PCR. Values represented as mean ± SEM where ***p ≤ 0.001 as compared to the vehicle control for AM630 and #### p ≤ 0.001 as compared to 2-AG.

mRNA expression of Collagen 1A and 3A in human ventricular fibroblasts after all the drug treatments was determined by quantitative real time PCR analyses. As seen in figure 20(A), collagen 1A mRNA expression was not significantly different when fibroblasts were treated with 2-AG as compared to the vehicle control. The CB2 antagonist, AM630, significantly elevated the mRNA levels of collagen 1A as compared to the vehicle control. The mRNA levels were also significantly elevated with the combined treatment of 2-AG + AM630 as compared to the vehicle. The expression of collagen 1A was significantly higher when the fibroblasts were co-treated with 2-AG + AM630 than the levels obtained with 2-AG alone. Collagen 3A mRNA levels were unaffected by all of the above drug treatments [figure 20(B)].

Figure 21: MCP-1 secreted by fibroblasts is altered by AM630 treatment

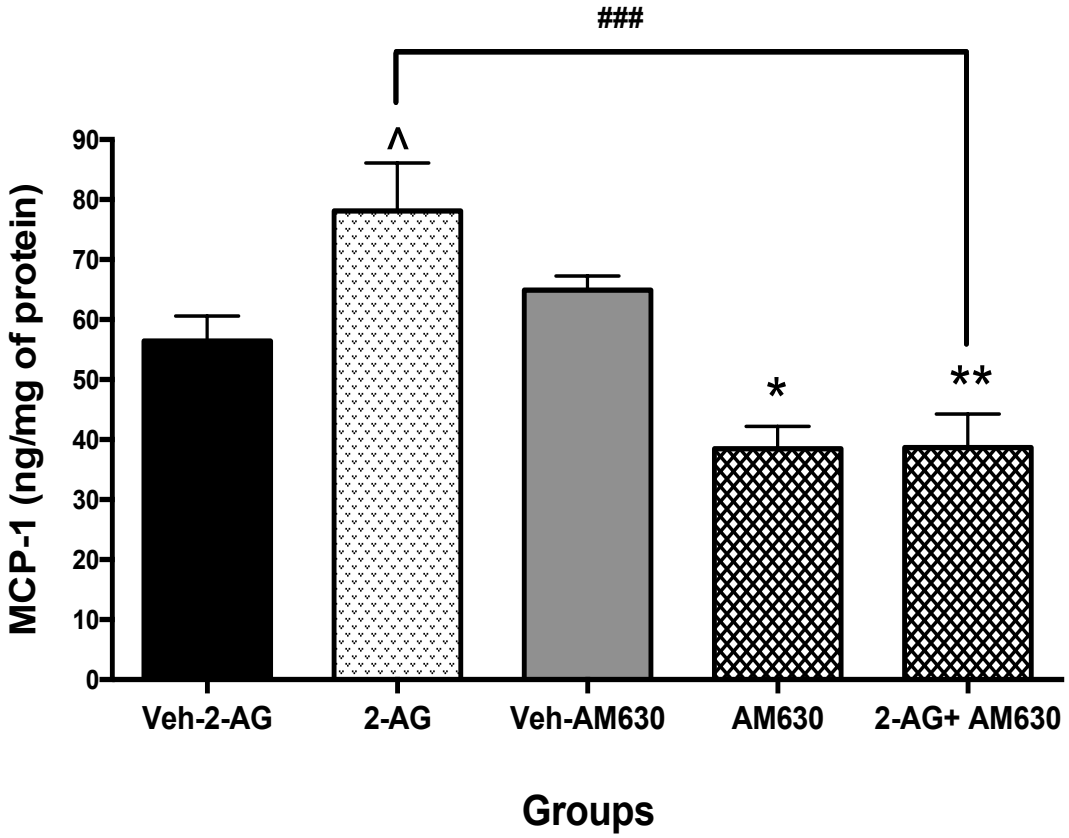


Figure 21: MCP-1 (ng/mg of protein) secreted in the culture media by human ventricular fibroblasts on treatment with 2-AG (10uM), AM630 (10uM) and 2-AG + AM630 (both 10uM) measured by Enzyme Linked Immunosorbent Assay. Values are represented as mean ± SEM where *p ≤ 0.05, **p ≤ 0.01 as compared to the vehicle control for AM630, ^p ≤ 0.05 as compared to the vehicle control for 2-AG and ###p ≤ 0.001 as compared to 2-AG.

MCP-1 secreted in the culture media by human ventricular fibroblasts was analyzed by a commercially available ELISA kit. MCP-1 secreted in the media was elevated by 45% on treatment with 2-AG as compared to the vehicle control (figure 21). The treatment of fibroblasts with the CB2 antagonist, AM630, caused a decrease of

about 42% in the amount of MCP-1 secreted in the media as compared to the vehicle control. MCP-1 secreted by the fibroblasts was also reduced by about a similar percentage as AM630 when co-treated by 2-AG + AM630 as compared to the vehicle. There was a significant decrease in MCP-1 levels obtained by combined treatment with 2-AG + AM630 as compared to those obtained by treating with 2-AG alone (figure 21).

DISCUSSION

The endocannabinoid system is well documented to play a role in various human disorders, including cardiovascular, neuropsychological, metabolic and inflammatory diseases (Pacher et al., 2006). This system is under extensive research for potential new therapeutic applications in such numerous areas. In the recent years, the endogenous (endocannabinoids) and exogenous (Δ^9-THC and synthetic cannabinoid compounds) ligands and the cannabinoid receptors have been implicated in cardiovascular disorders such as cardiomyopathy and ischemia involving cardiac remodeling. The CB2 receptors were initially found to be mainly expressed on immune cells, but were subsequently found to be expressed in the brain and peripheral organ systems (Onaivi et al., 2006; Pacher and Mechoulam, 2011). Later studies by various groups demonstrated the expression of these receptors in the different components of the cardiac system such as in human cardiomyocytes (Weis et al., 2010; Mukhopadhyay et al., 2010), human coronary artery smooth muscle cells (Rajesh et al., 2007) and vascular endothelial cells (Rajesh et al., 2008b) and rat cardiomyoblasts (Shmist et al., 2006). Here, we have shown the presence of CB2 receptors on human ventricular fibroblasts under basal conditions by immunofluorescence and western blotting using rabbit anti-CB2 polyclonal antibody. Binding to these receptors in immunofluorescence was confirmed by obtaining a negative control showing no signal

wherein the anti-CB2 antibody was not used. However, studies to date have not elucidated the effects of directly modulating these receptors on HVF and effects on production of collagen and the chemokine, MCP-1.

Total collagen secreted by the HVF in culture media was measured by the hydroxyproline content. An increase in total collagen secreted by the fibroblasts was seen on treatment with 2-AG by itself. The total collagen levels were significantly increased by the CB2 antagonist, AM630, and this elevation was seen even after fibroblasts were co-treated with 2-AG + AM630. Real time PCR analyses of expression of collagen 1A revealed that it was significantly increased by AM630 as well as by the combined treatment of 2-AG + AM630. Also, expression levels of collagen 1A obtained by co-treatment of 2-AG + AM630 were significantly higher than those obtained by 2-AG alone. Type 1A is a major type of collagen present in the ECM and increase in its expression could be the main contributor to increasing the total collagen synthesis by AM630. These results indicate that CB2 receptors seem to be involved in reducing collagen production and their antagonism exerts a profibrotic effect by enhancing collagen 1A expression, total collagen synthesis and secretion by HVF.

Previous studies have established that cannabinoids can produce antifibrogenic effects in a number of different organs via activation of CB2 receptors. CB2 activation was found to prevent fibrosis in the heart (Defer et al.2009) as well as in the liver (Julien et al., 2005; Munoz-Luque et al., 2008; Avraham et al., 2012), lungs and skin (Servettaz et al., 2010) and the pancreas (Michalski et al., 2008) by decreasing collagen production and accumulation and attenuating activation and proliferation of the HSCs in liver fibrosis. Defer and colleagues (2009) have demonstrated protective effects of CB2

receptors against post ischemia-reperfusion cardiac remodeling and heart failure. This study by Defer et al. (2009) provides evidence of improved survival of myocytes and fibroblasts in post ischemic hearts in CB2 null mice and a potential anti-fibrotic effect of CB2 receptors by reducing production of TGF-β.

We also measured levels of the chemotaxic factor, MCP-1, secreted in the culture media by HVF after antagonizing CB2 receptors with AM630. MCP-1 was found to be significantly elevated by treatment of 2-AG alone. Treatment with AM630 robustly decreased the amount of MCP-1 secreted by HVF. This decrease in MCP-1 levels was seen on treatment with AM630 even after HVF were stimulated by 2-AG in the combined treatment group. This indicates that CB2 receptors are involved in increasing production and secretion of MCP-1 by HVF. MCP-1 is known to induce collagen production and deposition (Dobaczewski and Frangogiannis, 2010). Thus, these results suggest that CB2 receptor antagonism is profibrogenic but this effect may be independent of MCP-1 expression. A previous study by Jbilo et al. (1999) had showed induction of MCP-1 gene expression by CB2 receptors in human promyelocytic cell line HL60. This effect was mediated via CB2 receptors coupled to G_i and was abolished by a CB2 antagonist. 2-AG acting via CB2 receptors was also able to increase expression of MCP-1 in the HL60 cell line (Kishimoto et al., 2004). Our results also show increased levels of MCP-1 by 2-AG and this effect was reversed by both, CB1 and CB2 antagonists. On the other hand, CB2 receptor activation has been shown to be anti-inflammatory and reduce the expression of MCP-1 induced by TNF in human coronary endothelial cells and in nephropathy (Rajesh et al., 2007; Mukhopadhyay et al., 2010b). Apparently, CB2 receptors can differently modulate MCP-1 expression in various cell

types but the studies vary in the usage of endocannabinoids or synthetic cannabinoids with different potencies and receptor specificities to obtain the said effects on MCP-1 (Lunn et al., 2006). Moreover, effects of CB2 receptors on immune system mediators have been shown to go against as well as the proinflammatory way as reviewed by Miller and Stella (2008).

CONCLUSION

In the current study, expression of CB2 receptors was demonstrated on HVF for the first time, to the best of our knowledge. CB2 antagonism was found to increase total collagen secreted and expression of collagen 1A by the HVF. However, blockade of CB2 receptors decreased the amount of chemokine, MCP-1, secreted by the HVF and hence seems to be independent of the effects seen on collagen production. Overall, in accordance with the current literature, our results indicate that CB2 antagonism is profibrotic and activation CB2 receptors could yield protective effects under fibrotic situations.

Table 5: Comparison of effects of CB1 and CB2 antagonism on HVF

Parameter	CB1 antagonism	CB2 antagonism
Total collagen	↓	↑
Collagen 1A	No change	↑
Collagen 3A	↓	No change
MCP-1	↓	↓

As seen in our current study, CB1 receptors seem to be involved in promoting collagen production and having a profibrotic effect while CB2 receptors seem to be producing an anti-fibrotic effect via reduction in collagen production. CB1 and CB2, thus, appear to modulate collagen production in an opposing manner and the resulting interplay between their actions seems to be one of the factors determining the levels of collagen produced and secreted by the human ventricular fibroblasts.

SUMMARY

The present study was designed to determine role of the endocannabinoid system in cardiac remodeling secondary to hypertension and effects of modulation of the cannabinoid CB1 and CB2 receptors on human ventricular fibroblasts on their downstream fibrotic responses. Rat model of pressure overload induced by abdominal aortic constriction was used for the *in vivo* study while the human ventricular fibroblast cell line was used for the *in vitro* study. The presence of CB1 and CB2 receptors was demonstrated on rat fibroblasts in left ventricular cross-sections and on human ventricular fibroblasts for the first time. The endocannabinoid system was found to be responsive in cardiac remodeling as the expression of CB1 and CB2 receptors was temporally alerted as the pathological changes progressed in the rat pressure overloaded hearts. CB1 receptor antagonism on human ventricular fibroblasts was found to decrease their collagen expression and production, thus having an anti-fibrotic effect while CB2 receptor antagonism was found to promote their collagen expression and production, thus having a profibrotic effect. Our results are in accordance with existing literature showing CB1 antagonism and CB2 agonism to be beneficial in various cardiovascular pathologies. Modulation of these receptors could thus offer an exciting new avenue for development of anti-fibrotic therapies and attenuating adverse remodeling changes.

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VITA

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