Quote

Knowledge Inspires Thoughts

Thoughts Reach Imagination

Imagination Leads to Creativity

Sherif Mahmoud
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University of Alberta

Drug-Disease Interaction: Effect of Inflammation on the Pharmacological Response to Calcium Channel Blockers

by

Sherif Hanafy Mostafa Hanafy MAHMOUD

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Examining Committee

Fakhreddin Jamali, Faculty of Pharmacy and Pharmaceutical Sciences

Dion Brocks, Faculty of Pharmacy and Pharmaceutical Sciences

Ayman El-Kadi, Faculty of Pharmacy and Pharmaceutical Sciences

John Seubert, Faculty of Pharmacy and Pharmaceutical Sciences

Maury Pinsk, Faculty of Medicine, Pediatric Nephrology

Daniel S. Sitar, Faculty of Medicine, Pharmacology and Therapeutics, University of Manitoba

Dedication

To my lovely wife Sally, for being my soul

To my lovely Mom and Dad, for setting the stage for my life

To My wonderful Mom and Dad-in law, for their continuous support

To My precious Basant and Omar, for being the pearls of my life

To Dr Jamali, for his guidance to my scientific maturity

Abstract

The present research is focused on the topic of inflammation-drug interaction. Inflammation complicates many human diseases and conditions ranging from obesity to cancer. Therefore, the study of the effect of inflammation on drug pharmacokinetics and pharmacodynamics is pivotal. First, we tested the hypothesis that controlling inflammation using valsartan can restore the previously reported altered verapamil pharmacokinetics and pharmacodynamics. Such an effect is expected due to the anti-inflammatory properties of angiotensin II inhibition. Inflammation resulted in L-type calcium channel target protein ($Ca_v1.2$) downregulation and reduced verapamil potency in pre-adjuvant arthritis rat model. Valsartan treatment reversed the observed downregulation of L-type calcium channels thereby enhancing verapamil potency. This beneficial interaction, once proven in humans, may be of value in cardiac patients with superimposing inflammatory diseases. Second, we investigated whether the response to verapamil is reduced in experimentally induced acute myocardial injury (AMI) in rats. AMI caused a 75% reduction in verapamil potency and $Ca_v1.2$ target protein downregulation. If extrapolated to humans, our observations may suggest that L-type calcium channel downregulation can contribute, at least in part, to the poor outcome in myocardial infarction patients treated with calcium channel blockers (CCBs). Third, we studied the effect of obesity on the pharmacological response of CCBs in children with renal disease. Our data indicated that obese children are less responsive to CCBs than non-obese ones. Therefore, obesity should be considered when initiating antihypertensive drug

therapy in children. Last, we were interested in finding out if the expression of other target genes is also altered by inflammation. We used real time polymerase chain reaction, after determination of the best housekeeping gene to be used as an internal control. Inflammation resulted in significant alterations of several molecular targets and transporters affecting the pharmacokinetics and pharmacodynamics of drugs. These findings may provide an insight into the effect of inflammation on drug targets and modulators of disease pathogenesis. In conclusion, inflammation is a missed ring in the chain of therapy. The research presented in this thesis will add to the inflammation-drug interaction field important findings that will help understanding the role of inflammation in pharmacotherapy outcomes.

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Table of contents

List of Tables

List of figures

Figure 6-15. Effect of adjuvant arthritis on ACE-2/ACE constitutive gene expression ratio in different rat organs (n=4/group). *, $p<0.05$ vs. control rats. 181

List of Abbreviations

AA	Adjuvant arthritis
AAG	α1-acid glycoprotein
ACE	Angiotensin converting enzyme
ACE-2	Angiotensin converting enzyme 2
ACEIs	Angiotensin converting enzyme inhibitors
AGI	Angiotensin I
AGII	Angiotensin II
AMI	Acute myocardial infarction
ANGI	Angiotensin interrupting agents
APC	Antigen presenting cell
ARB	Angiotensin II receptor blocker
AT1	Angiotensin II receptor 1
AT2	Angiotensin II receptor 2
AUC	Area under the curve
AUEC	Area under the effect curve
AV node	Atrioventricular node
BCRP	Breast cancer resistance protein
BCS	Biopharmaceutical classification system
BDDCS	Biopharmaceutical drug disposition classification system
BMI	Body mass index
BSEP	Bile salt export pump
$Ca_v1.2$	alpha1C subunit of L-type calcium channel

CCBs	Calcium channel blockers
Cmax	Maximum drug concentration
COX1	Cyclooxygenase 1
COX2	Cyclooxygenase 2
CRP	C-reative protein
cTnI	Cardiac troponin I
CYP450	Cytochrome P450
DHP	Dihydropyridine
ECG	Electrocardiogram
FAD	Flavin adenine dinucleotide
FMO	Flavine monooxygenase
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
G-CSF	Granulocyte colony stimulating factor
GM-CSF	Granulocyte macrophage colony stimulating factor
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HK	Housekeeping
HPLC	High performance liquid chromatography
HR	Heart rate
ICAM-1	Intracellular adhesion molecule-1
ICD-9	International classification of diseases version 9
IFN	Interferon
IL	Interleukin
iNOS	Inducible nitric oxide

LAT	L-amino acid transporter
MCP-1	Monocyte chemoattractant protein-1
MCT	Monocarboxylate transporter
MDR	Multidrug resistance transporter
MHC	Major histocompatibility complex
MRP	Multidrug resistance-associated protein
NADPH	Nicotinamide adenine dinucleotide phosphate
NKC	Natural killer cells
OAT	Organic anion transporter
OATP	Organic anion transporter protein
OCT	Organic cation transporter
OCTN	Organic cation/carnitine transporter
PAF	Platelet activating factor
PAI-1	Plasminogen activator inhibitor-1
PEG400	Polyethylene glycol 400
PEPT	Peptide transporter
PG	Prostaglandin
PKA	Protein kinase A
PKC	Protein kinase C
PMN	Polymorphnuclear leukocytes
RA	Rheumatoid arthritis
RAS	Renin angiotensin system
ROS	Reactive oxygen species

SA node	Sinoatrial node
SD	Standard deviation
SEM	Standard error of the mean
SERCA	Sarco/endoplasmic reticulum calcium ATPase
SLE	Systemic lupus erythematosus
Th1	T-helper cell 1
Th2	T-helper cell 2
TNF-α	Tumor necrosis factor alpha
VGCC	Voltage-gated calcium channels

Chapter One

1. Introduction

Inflammation is the physiological reaction of the body towards external and internal stimuli. It is associated with the release of cytokines and other inflammatory mediators which have been found to alter the pharmacokinetics and pharmacodynamics of many drugs in humans[1-3] and animals[4-6]. This may be responsible, at least, in part for the reduced outcome and prognosis in inflammatory diseases[7].

Verapamil, a phenylalkylamine calcium channel blocker, is an example of those drugs that have altered pharmacokinetics and pharmacodynamics in inflammation[4, 8]. It has been found that the plasma verapamil concentration is significantly elevated in rheumatoid arthritis patients[3]. Despite its increased concentration, verapamil potency is reduced under inflammatory conditions, an effect that has been explained by reduced binding to L-type calcium channels in the heart[4]. Valsartan, an angiotensin II receptor blocker, has been found to reverse the diminished verapamil-induced PR-interval prolongation in inflammation[9]. We studied the mechanism behind the interaction and determined whether this effect is also associated with normalization of the altered verapamil pharmacokinetics in pre-adjuvant arthritis rats. In addition to inflammatory diseases, inflammation complicates a tremendous number of conditions such as hypertension[10], acute myocardial infarction[11], heart failure[12] and obesity[13, 14]. Therefore, we were interested to study the effect of inflammation on the pharmacodynamics of

calcium channel blockers in experimentally-induced myocardial injury and childhood obesity, two conditions associated with inflammation. Then we characterized the effect of inflammation on the molecular targets and transporters affecting the pharmacokinetics and pharmacodynamics of drugs to help gain insight into the role of inflammation in pharmacotherapy outcomes.

1.1. Inflammation

Inflammation is a physiological response to many endogenous and exogenous stimuli. Literally, inflammation means burning as it reflects its characteristics: redness, heat, swelling and pain. Inflammation is the normal defense mechanism that the body uses to defend it from infections and foreign bodies. Moreover, it facilitates wound healing by removing invading pathogens and cellular debris helping the survival of the organism. However, the inflammatory response may become exaggerated causing harm to the body[15]. In addition, a chronic inflammatory response may lead to several autoimmune diseases such as rheumatoid arthritis, systemic lupus erythematosus and multiple sclerosis. The inflammatory response is produced as a result of two immune mechanisms acting in concert, innate and adaptive immunity[16, 17].

Innate immunity is the natural immunity that provides a non-specific protection against invading organisms. It involves physical mechanisms such as the skin and mucosal lining of the gastrointestinal tract that prevent invading organisms from entry into the body, chemical mechanisms such as the secretion

of lysozymes in tears and saliva and cellular defense mechanisms[18]. The latter is activated by binding of receptors to common sequences of amino acids, sugars or fats on the invading bacteria leading to phagocytosis by tissue macrophages and neutrophils, release of bactericidal enzymes and interferons, activation of the complement system and mast cell degranulation[16, 18]. Adaptive immunity is the immunity that develops against specific antigens. It has both humoral and cellular components. The humoral component includes the antibodies that are secreted from plasma cells (derived from B lymphocytes) and a cellular component that includes T lymphocytes[16, 17]. A cross-talk exists between innate and adaptive immunity. Active macrophages release some cytokines like interleukin-1 (IL-1) and IL-6 that recruit and activate lymphocytes. Moreover, antigen presenting cells (APCs) such as dendritic cells and macrophages digest the antigen and couple its product to its surface major histocompatability complex (MHC). This can be detected by naïve lymphocytes which are then activated to produce T cells and B cells. B-cells produce immunoglobulins (antibodies) that are antigen-specific. T cells are subdivided into CD8+ (Killer T cells) which are cytotoxic and kill infected and foreign cells, and CD4+ T helper cells (Th) that modulate the immune response. CD4+ T helper cells are further subdivided into Th1 and Th2. Functional balance between Th1 and Th-2 determines the severity of inflammation and the degree of tissue damage[19, 20]. After the resolution of inflammation some T and B cells persist as memory cells that can detect further exposure to the antigen.

Inflammation can be either acute or chronic. In acute inflammation, following acute tissue injury or infection, the local blood vessels dilate to increase blood supply to the area. Neutrophils are attracted to the site of insult by the release of chemotactic factors such as the activated complement factor C5a, platelet-activating factor (PAF) and leukotrienes. This is followed by binding of neutrophils to the newly expressed endothelial surface adhesion molecules (Selectins) and infiltration into the tissues by diapedesis. Neutrophils and tissue macrophages phagocytose the invading organism. In addition, reactive oxygen species (ROS), myeloperoxidase and metalloproteinases are released from neutrophils with added toxic effects on the bacteria and the surrounding tissues[16].

Chronic inflammation is a condition in which inflammation persists for prolonged periods as result of failure of immune cells to eliminate the injurious agent or when the stimulant is persistent like in *Mycobacterium tuberculosis* infections and chronic exposure to exogenous particles such as carbon dust[21]. Chronic inflammation occurs as a result of specific antigen-activated T cells-induced proliferation of macrophages or a non-specific innate immune mechanism[17]. Activated macrophages secrete many mediators like coagulation factors, cytokines, ROS and arachidonic acid derivatives that modulate the inflammatory response. This is also coupled with the activity of other inflammatory cells like activated T and B lymphocytes and eosinophils. The chronic inflammatory reaction can also develop against the host's own tissue leading to a large array of autoimmune diseases[17, 18, 21]. Chronic inflammation can

be granulomatous like in tuberculosis, leprosy, crohn's disease and sarcoidosis or non-granulomatous such as chronic viral hepatitis, rheumatoid arthritis, Hashimoto's thyroiditis, and ulcerative colitis[22]. Moreover, chronic inflammation complicates many other diseases and conditions such as obesity[13, 14], cardiovascular diseases[10-12] and cancer[7]. That is why chronic inflammation is involved in most human diseases.

The development and progression of inflammatory conditions are mainly modulated by cytokines. Originally known as lymphokines and monokines, cytokines are molecules that are secreted mainly from lymphocytes and macrophages. They are also secreted from other cell types such as glial cells and endothelial cells[16]. By acting through an autocrine, paracrine and/or endocrine fashion, cytokines are considered the main modulators of the inflammatory response and the means by which cells affect each other. Cytokines have several and different functions that may be unique, overlapping, pro-inflammatory, anti-inflammatory, beneficial or harmful[17]. There are more than 100 cytokines discovered to date[16]. Cytokines include interleukins (numbered according to their discovery from IL-1 to IL-35), interferons, colony stimulating factors, tumor necrosis factors and chemokines[17, 23]. Chemokines are a group of chemicals secreted from endothelial cells and macrophages and function in recruiting and attracting other immune cells to the site of inflammation[17]. Table 1-1 depicts a select cytokines of importance and their biological role in the immune system.

Table 1-1. Selected Cytokines of importance, their biological function and clinical importance. Adapted from references[16, 17, 23, 24]

Cytokine Name	Source	Biological function/ clinical importance
Interleukins (IL)		
IL-1 (IL1α and IL1β)	Macrophages	Activates T-cells and macrophages Implicated in the pathogenesis of RA, septic shock and atherosclerosis
IL-1ra	Macrophages	IL-1 antagonist
IL-2	Th1	Activates NKC, macrophages and lymphocytes Used in the treatment of metastatic renal carcinoma and melanoma
IL-4	Th2, NKC and mast cells	Stimulates IgE production Activates lymphocytes and monocytes Plays a role in allergy
IL-6	Macrophages	Activates lymphocytes and B cells Involved in acute phase protein production Involved in the pathogenesis of myeloma and mesangial proliferative glomerulonephritis
IL-10	Th2	Inhibits cell mediated inflammatory responses
IL-12	Dendritic cells,	Stimulates production of IFNγ

	B cells and macrophages	Stimulates Th1 response
Tumor Necrosis Factors (TNF)		
TNF-α	B cells, Th1, NKC and mast cells	Antibodies against TNF-α used in the treatment of autoimmune diseases like RA
Interferons (IFN)		
IFN-α & IFN-β	Cells infected with viruses	Promotes resistance to viral infections Used for treatment of Hepatitis B and C infections IFN-β is used for control of multiple sclerosis
IFN-γ	Th1 and NKC	The main macrophage activator Inhibitor of Th2 response
Colony Stimulating Factors (CSF)		
GM- CSF	B cells, endothelial cells, macrophages, fibroblasts, NKC and T-cells	Promotes growth of granulocyte and macrophage precursors Used in the treatment of neutropenia Stimulates cell production after bone marrow transplants

G-CSF	Monocytes, granulocytes, fibroblasts and some tumor cells	Promotes growth of granulocytes
Chemokines		
MCP-1	Macrophages and endothelial cells	Promotes monocytes and T cells chemotaxis to site of inflammation

RA, rheumatoid arthritis; NKC, natural killer cells; Th, T-helper cells; MCP-1, monocyte chemoattractant protein.

The development of the immune response to a specific infection requires the activation of particular immune mechanisms and cytokine release profiles to allow elimination of the injurious agent. For example, while elimination of bacteria and viruses requires cell-mediated immunity, elimination of toxins requires activation of the humoral immunity. It has been found that the cytokine release profile mediated by Th1 and Th2 is the main determinant of the type of immune response developed[25]. Th1 secretes mainly pro-inflammatory mediators such as interferon gamma (IFN-γ), tumor necrosis factor alpha (TNF-α) and IL-2. It modulates cell-mediated immunity, macrophage activation and excessive tissue injury. On the other hand, Th2 mainly secretes anti-inflammatory mediators such as IL-4 and IL-10 and mediates humoral immunity, eosinophil activation and allergic reactions[17-19]. Activation of either T-helper cells suppresses the other leading to polarization of the immune response towards specific sets of immune reactions. Th1/Th2 polarization has been found to be involved in the pathogenesis of many human diseases. For example rheumatoid arthritis, multiple sclerosis and type I diabetes mellitus result from Th1 overactivation with increased production of pro-inflammatory mediators. On the other hand, Th2 overactivation has been implicated in the development of allergy, systemic lupus erythematosus, hay fever, progressive systemic sclerosis and eczema[25, 26].

In addition to their function in immunomodulation and immunity-related diseases, cytokines, through their actions on cell receptors, can alter the phenotype, function and/or gene expression of non-immune cells leading to a

huge array of effects, and in some case adverse effects, that are responsible for the involvement of inflammation in the pathogenesis and prognosis of human diseases. Binding of cytokines to their respective receptors on hepatocytes can alter transcription and translation processes and resultant protein products[27] such as altered production of metabolic enzymes and acute phase proteins. Moreover, inflammatory cytokines have been implicated in the downregulation of contractile proteins in cardiac myocytes such as alpha myosin heavy chain contributing to heart failure[28].

In addition to cytokines, nitric oxide (NO) plays an important role in inflammation. NO is produced from the amino acid L-arginine by the action of the enzyme nitric oxide synthase (NOS). Three forms of NOS have been discovered, endothelial (eNOS), neuronal (nNOS) and inducible (iNOS). eNOS and nNOS are constitutively expressed and are responsible for the formation of NO under physiological conditions to perform vital functions such as vasodilatation and neurotransmission. Downregulation of eNOS and subsequent reduced NO production causes endothelial dysfunction and harmful vascular effects. On the other hand, iNOS is not constitutively expressed; however, its expression is increased in inflammatory conditions by the action of pro-inflammatory cytokines such as IL-1α, IFN-γ and TNF-α leading to NO overproduction and subsequent harmful effects[29]. NO has been found to play a role in the pathogenesis of many diseases such as septic shock[30], rheumatoid arthritis[31], acute myocardial infarction[32], glomerulonephritis[33] and congestive heart failure[34]. It promotes tissue

injury, joint destruction, vascular unresponsiveness, cellular apoptosis, Th1/Th2 imbalance and cardiodepression[30, 31, 35, 36]. It has been found that the level of serum nitrite, a stable breakdown product of NO, is correlated with the severity of rheumatoid arthrtis[3]. In addition to promoting inflammation and tissue injury, overexpressed NO has been found to alter the expression of drug metabolizing enzymes[37]. Hepatic cytochrome P450s expression has been found to be inversely correlated with serum nitrite in arthritis rat model[8], suggestive of its role in inflammation-drug interaction.

1.2. Inflammatory Conditions

Inflammation plays an important role in the pathogenesis and progression of a broad range of diseases and conditions. They include inflammatory diseases such as rheumatoid arthritis, systemic lupus erythematosus, Crohn's disease, ulcerative colitis, asthma and dermatitis. In addition, inflammation complicates other diseases and conditions such as acute myocardial infarction, hypertension, heart failure, obesity and aging. Table 1-2 depicts a list of human conditions where inflammatory mediators are involved. In the present work we were interested in arthritis, acute myocardial infarction and obesity.

Table 1-2. List of human conditions associated with altered inflammatory mediator profiles. Adapted from reference[38].

Condition	Cytokine alteration
Human Immunodeficiency Virus (HIV)	High viral load is associated with increased secretion of TNF-α, IL-1 and IL-6 from monocytes and macrophages; increased IL-10 expression and B-cell hyperactivity are associated with increased risk of HIV infection-associated lymphoma.
Infection	Severe bacterial infections are associated with increased IL-6 and myeloperoxidase; malaria infection is associated with increased TNF-α production; sepsis is considered an acute inflammatory state characterized by the release of a great number of pro-inflammatory mediators such as TNF-α and IL-6; tuberculosis osteomyelitis is associated with elevated IL-6 concentration.
Acute Myocardial Infarction (AMI)	Concentrations of TNF-α, IL-6, IL-1β are elevated following AMI; increased CRP concentration is an important predictor of AMI.

Hypertension	Hypertension is associated with increased IL-1ra production.
Allergic reactions	Th2 overactivation (increased IL-4, IL-5 and IL-10) is an important feature of allergic reactions.
Behçet's disease	Disease activity is associated with increased IL-6, IL-10, IL-17, IL-18 and IFN-γ.
Prostate Cancer	IL-6 is correlated to disease metastasis.
Multiple Myeloma	Increased IL-6 is correlated with increased mortality.
Atherosclerosis	Its pathogenesis is an inflammatory process associated with P-selectin, VCAM-1 and ICAM-1 accumulation.
Heart Failure	Increased IL-6 and TNF-α concentrations are associated with disease progression, worsening and increased mortality.
Stroke and brain ischemia	It is associated with iincreased IL-1, TNF-α, TNF-β and IL-6 concentrations; reduced IL-10 level is associated with poor stroke prognosis.
Obesity	Chronic low grade inflammatory state (increased CRP, TNF-α, IL-6 and IL-8)
Unstable angina	It is associated with increased pro-inflammatory mediator concentrations; IL-6 concentration is positively correlated with increased mortality.

Fever	It is associated with increased IL-1α, 1β, TNF-α and IL-6 production.
Aging	Chronic low grade inflammatory state (increased IL-1, TNF-α and IL-6).
Peptic Ulcer	Ulcer pathogenesis and recurrence have been linked to the pro-inflammatory mediators TNF-a, IL-1 and IL-8.
Crohn's Disease	It is associated with Th1 hyperactivity.
Diabetes Mellitus	TNF-α associated obesity and diabetes is responsible for insulin resistance.
Hepatic Disease	TNF-α plays a role in the pathogenesis non-alcoholic steatohepatitis; IL-18 and IL-18 binding protein levels are related to chronic hepatic disease severity.
Alzheimer's Disease	Increased IL-1, TNF-α and IL-6 concentrations participate in the disease pathogenesis through neuronal upregulation of acetylcholinesterase and the neurodegenerative amyloid-β.
Rheumatoid Arthritis	It is associated with increased TNF-α, IL-1, IL-6, MCP-1, GM-CSF and IL-8 concentrations.
Thyrotoxicosis	It is associated with increased IL-6 and IL-8 concentrations.

Multiple Sclerosis	It is associated with increased expression of pro-inflammatory mediators and adhesion molecules in the central nervous system and in the periphery.
Pain	Many pro-inflammatory mediators have been implicated in pain induction peripherally and centrally such as IL-6, TNF-α and IL-1.
Psychiatric disorders	Delirium-complicated conditions are associated with elevated concentrations of interferons and interleukins; obsessive compulsive disorder patients had reduced plasma levels of IL-1β and TNF-α; schizophrenia and depression are associated with elevated levels of IL-6 and TNF-α.
Burn	Elevated IL-6 concentrations are associated with increased mortality rates among burn patients.
Stress	It is associated with elevated IFN-γ, TNF-α, IL-1, IL-6 and IL-1ra and reduced IL-4 and IL-10 concentrations (Th1 polarization).
Pancreatitis	Elevated MMP-1, and TNF-α concentrations can predict poor disease prognosis and higher mortality rate; inflammatory mediators plays an important role in the pathogenesis of the disease such as IL-1β, IL-6, IL-8, TNF-α, PAF, ICAM-1,

	IL-10 and C5a
Organ Transplant	Elevated concentrations of TNF-α, IFN-γ and IL-1ra are associated with poor prognosis in hepatic, renal and lung transplant patients, respectively; Th2 polarization favors liver transplant in infants

TNF, tumor necrosis factor; IL, interleukin; CRP, C-reactive protein; IFN, interferon; VCAM, vascular cell adhesion molecule; ICAM, intercellular adhesion molecule; MCP-1, monocyte chemoattractant protein 1; GM-CSF, granulocyte monocyte colony stimulating factor; MMP-1, matrix metalloproteinase-1; PAF, platelet activating factor; C5a, activated complement 5.

1.2.1. Rheumatoid Arthritis

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by autoimmune joint inflammation, swelling, pain and eventually joint deformity. Not only is RA confined to joints, but also extra-articular involvements are not uncommon. This includes cardiovascular diseases, rheumatoid nodules, ocular inflammation, pulmonary diseases, neuropathy, splenomegaly and thyroid hormone alterations. Rheumatoid arthritis is more common in females than males. It affects 1% of the population and its incidence increases with increasing age with onset usually from forties to sixties[18].

The pathogenesis of rheumatoid arthritis is not well understood. However, it develops as a result of the loss of immune tolerance towards self antigens. Activated B cells produce antibodies against joint tissues leading to complement system activation, polymorphonuclear leukocytes recruitment, ROS and other cytotoxic chemicals release, phagocytosis and antigen presentation to T cells. T cell activation by APCs is considered a key element in the pathogenesis of RA. A polarized Th1 response has been found in RA. Activated Th1 cells produce the pro-inflammatory mediators TNF-α and IL-1 that are responsible for the inflammatory reaction and activation of macrophages and more T cells. Joint inflammation subsequently leads to joint tissue proliferation (pannus formation) and invasion of the surrounding cartilage and bone leading to joint destruction[18, 39].

Rheumatoid arthritis patients have demonstrated higher mortality rates as compared to general population[40-43]. This is mainly due to increased cardiovascular complications in those patients[43-45]. They have increased incidence of cerebrovascular atherosclerosis, heart failure and acute coronary syndromes[46]. The degree of systemic inflammation is considered as one of the main factors contributing to adverse cardiovascular events and increased mortality in those populations[18, 42]. Inflammation-induced endothelial dysfunction combined with impaired vascular repair in RA patients accelerate the development of premature atherosclerosis[45]. The immunological mechanisms involved in the pathogenesis of atherosclerosis have been found to resemble those happening in RA development[47]. Elevated levels of TNF-α, IL-1, IL-6, IL-17, CRP, MCP-1 and cellular adhesion molecules such as vascular cell adhesion molecule-1 (VCAM-1) and intracellular adhesion molecule-1 (ICAM-1) in RA patients have been found to be risk factors for cardiovascular disease-related mortality. They are also promoters of the development of atherosclerosis and vascular dysfunction[45, 46, 48]. In addition, TNF-α, IL-6 and IL-17 promote insulin resistance[46], another important risk factor for cardiovascular complications[49]. Another marker of the inflammatory severity in RA patients is the blood level of $CD4^{+}CD28^{-}$ T cells where elevated counts have been found to be injurious to the endothelium and accelerate vascular dysfunction[50]. In addition to atherosclerosis and increased risk of thrombo-embolism, other factors such as lipid abnormalities, thyroid dysfunction, decreased physical activity resulting from the disabling nature of the disease may contribute to the adverse cardiovascular complications[18, 51].

Interestingly, it has been found that some of the population cardiovascular risk factors such as male gender and smoking have less impact on the incidence of cardiovascular events in RA patients as compared to the general population. This suggests that preventing cardiovascular complications should not be only focused on the controllable classic risk factors such as smoking and hyperlipidemia without controlling the non traditional risk factors associated with the disease itself such as systemic inflammation[41]. In addition of being a risk for cardiovascular complications, inflammation has been found to reduce potency of some commonly used cardiovascular drugs such as verapamil and propranolol by downregulating their target protein[3, 52]. This further complicates the therapy in RA patients who have an increased need for those drugs. For example up to 50% of RA patients have been reported to suffer from hypertension[41].

1.2.2. Acute Myocardial Infarction

Acute myocardial infarction (AMI) is a serious consequence of ischemic heart diseases with narrowed coronary blood vessels. AMI and unstable angina, known collectively as acute coronary syndromes, are responsible for the major part of cardiovascular disease-related mortality which is considered the leading cause of death across the globe[18]. Acute myocardial infarction occurs when myocardial oxygen supply is severely diminished. This is mainly due to coronary arteries occlusion by clotting or less commonly coronary embolism, severe vascular spasm or increased cardiac metabolic demands. Coronary blood clot develops as a result of splitting or erosion of the atherosclerotic plaque lining the blood vessel.

This is followed by platelet recruitment and aggregation and activation of the clotting factors[18].

Inflammation plays a major role in the pathogenesis and prognosis of acute myocardial infarction[53-56]. Inflammation has been implicated in the initiation and development of the atherosclerotic plaque, the main culprit of AMI[54]. Numerous risk factors such as cigarette smoking, diabetes mellitus, hyperglycemia and elevated blood pressure can induce endothelial cellular injury leading to increased expression of adhesion molecules and chemotactic chemicals that recruit blood monocytes, macrophage precursors, into the injury site. Activated macrophages produce various pro-inflammatory mediators that recruit other inflammatory cells such as mast cells and T cells and phagocytose the atherogenic lipoproteins leading to lipid accumulation. In addition, macrophages secrete various cytokines such as TNF-α and metalloproteinases that cause collagen lysis and smooth muscle cells apoptosis. Collagen lysis weakens the atherosclerotic plaque making it prone to rupture, erosion and further clotting[41].

Inflammation also plays a role in myocardial injury following ischemia and reperfusion. From the initial stages of ischemia, an inflammatory cascade of events is initiated such as complement system activation, neutrophils attraction to the site of ischemia and release of reactive oxygen species that leads to tissue damage. This was followed by recruitment of T cells to the site of inflammation[53, 55]. Functional imbalance in Th1/Th2 ratio has been reported in AMI patients

leading to elevation of inflammatory mediator (e.g. TNF-α, IL-8 and IL-6) concentrations[25, 44]. The latter has been associated with poor prognosis and extended hospital stay[57, 58]. Similarly, post-ischemic injury and tissue perfusion have been implicated by local inflammatory reactions that further compromise the prognosis of the insult[56]. The involvement of inflammation in the different stages of acute myocardial infarction provides an insight on the pathophysiology of the disease and the need for appropriate means to control it. At the same time, inflammation-complicated AMI may alter the action and disposition of the drugs used in its managements complicating the disease prognosis.

1.2.3. Obesity

Obesity is one of the major health problems affecting our world. In Canada, the obese, defined as body mass index (BMI) $\geq$ 30 kg/m^2, represents about a quarter of the population. It is considered a result of the interplay between environmental, social and hereditary factors leading to "positive energy balance" and body fatness[59]. Obesity is considered a major risk factor for many diseases such as cardiovascular diseases, diabetes, osteoarthritis and cancer (Table 1-3)[59]. It can affect all age groups including pediatric population. The incidence of childhood obesity in North America has increased dramatically over the past three decades[60-63]. In Canada, the prevalence of pediatric obesity has tripled in 20 years[60]. Obesity in children is considered a risk factor for adult cardiovascular diseases[64] and is associated with poor disease outcomes[65].

Obesity has been regarded as an inflammatory process[13, 66] associated with chronic low grade inflammation[13, 14]. The main source of inflammatory mediators in obese individuals is adipose tissue[67] infiltrated by immune cells such as macrophages, granulocytes and lymphocytes[66, 68, 69]. This has been associated with increased secretion of IL-6, TNF-α, MCP-1, angiotensinogen and plasminogen activator inhibitor-1 (PAI-1)[69]. Moreover, CRP concentration, a major cardiovascular risk factor, is positively correlated to the degree of obesity[14, 66, 70]. Similar to adults, overweight and obese children have elevated inflammatory mediator levels such as CRP and TNF-α as compared to the general pediatric population[14, 71-73]. Inflammation is considered the link between obesity and insulin resistance and atherosclerosis[74, 75]. Insulin resistance observed in obese individuals has been attributed to TNF-α-mediated inhibition of insulin receptor substrate-1 phophorylation, an important step in insulin signaling[75]. Atherosclerosis, an inflammatory process induced by various inflammatory mediators, can be induced by the chronic inflammatory state observed in obesity[74]. Moreover, obesity is associated with increased secretion of the atherogenic leptin and resistin and reduced secretion of the anti-inflammatory adiponectin that is normally secreted from adipose tissues under physiological conditions[76].

Obesity is an independent risk factor for high blood pressure in adults. The prevalence of high blood pressure in adults increases from 15% in subjects with BMI < 25 kg/m^2 to up to 42% in ones with BMI≥ 30 kg/m^2 [77]. Similar to obesity,

inflammation complicates hypertension. Chae *et* al has reported a positive correlation between systolic blood pressure and the circulating levels of IL-6 and the intracellular adhesion molecule-1 (ICAM-1) which helps recruitment and migration of neutrophils across the endothelial layer[78]. In addition, elevated CRP, MCP-1, TNF-α and P-selectin have been observed in individuals with elevated blood pressure[78]. It appears that there is a bidirectional relationship between hypertension and inflammation. Inflammation contributes to the development of hypertension and at the same time the latter induces inflammation by increasing tension on blood vessel walls and mediating atherosclerosis[10]. Similar to adults, obese children have a three-fold increased risk of hypertension as compared to non obese[79].

The incidence of pediatric hypertension has been reported to range from 0.8 to 5%[80]. Since the clinical outcome from uncontrolled hypertension may target various organs and results in imminent damage, clinical, pharmacological and non-pharmacological measures need to be instituted. Antihypertensive drug therapy is indicated in children whose hypertension is secondary, symptomatic, persistent despite life style modifications and/or associated with target-organ damage such as left ventricular hypertrophy[81]. Table 1-4 depicts commonly used antihypertensive agents and their recommended doses. Poor disease outcome is a common feature among obese population such as in organ transplants[65], breast cancer[82], ischemic heart diseases[83] and bacteremia[84]. This may be explained in part due to reduced efficacy of drugs in obesity. For example, it has been found

that obese adults have reduced response to verapamil and antidepressants as compared to non-obese[1, 85]. If extrapolated to pediatric population, the efficacy of antihypertensive drug therapy in obese children may not be similar to non obese especially the current recommendations to treat high blood pressure in obese children are the same as non-obese[81].

Table 1-3. Complications of obesity. Adapted from reference[86].

Cardiovascular diseases	Hypertension, myocardial hypertrophy, atherosclerosis
Metabolic diseases	Diabetes mellitus, hypertriglyceridemia, hypercholesterolemia
Gastrointestinal diseases	Biliary stones, fatty liver
Rheumatic diseases	Osteoarthritis
Skin diseases	Fungal infections, dermatitis
Cancer	Prostate, endometrial, breast and colon cancer
Respiratory diseases	Sleep apnea, asthma

Table 1-4. Commonly prescribed antihypertensive agents in pediatric patients and their recommended doses. Adapted from references[80, 81, 87].

Drug Category	Drug name	Dose
ACE inhibitors		
	Captopril	0.9–6 mg/kg/day in three divided doses
	Enalapril	0.08-0.6 mg/kg/d
	Lisinopril	0.07-0.6 mg/kg/d
Angiotensin-receptor blockers		
	Losartan	0.7-1.4 mg/kg/d
	Valsartan	2 mg/kg/d
ß-Blockers		
	Atenolol	0.5–2 mg/kg/d
	Metoprolol	1–6 mg/kg/d
	Propranolol	1–4 mg/kg/d in 2-3 divided doses
Calcium channel blocker		
	Amlodipine	6–17 years: 2.5–5 mg/d
	Nifedipine long acting	0.25-3 mg/kg/d
Central α-agonist		
	Clonidine	>12 yrs 0.2-2.4 mg/d
Diuretics		
	Hydrochlorothiazide	1-3 mg/kg/d

	Furosemide	0.5–6 mg/kg/d
	Spironolactone	1-3.3 mg/kg/d
Vasodilators		
	Hydralazine	0.75-7.5 mg/kg/d
	Minoxidil	<12 yrs: 0.2 mg/kg/d
		>12 yrs 5-100 mg/d

1.3. Inflammation-Drug interaction

Inflammation complicates many diseases and conditions such as rheumatoid arthritis, obesity, and acute myocardial infarction. Not only is inflammation involved in the pathogenesis and presentation of diseases, but also it affects the fate of the drugs in the body at both the pharmacokinetic and pharmacodynamic levels.

1.3.1. Effect of Inflammation on Pharmacokinetics of Drugs

It has been found that inflammation can alter the pharmacokinetics of drugs at all levels of absorption, distribution, metabolism and excretion.

Absorption of orally administered drugs is a process that is dependent on the interplay among drug, dosage form characteristics and the gastrointestinal tract physiology. Factors such as gastric emptying, gastric and intestinal pH, mucosal integrity, transporter function and intestinal peristalsis can affect drug absorption. Systemic and local intestinal inflammation have been found to alter some of these processes leading to altered drug absorption, rate and/or extent. For example, administration of lipopolysaccharides in rats and mice led to delayed gastric emptying and subsequent delay in salicylate absorption[88]. Intestinal inflammation observed in inflammatory bowel diseases is associated with altered drug permeability, an effect mediated by cytokines. While some cytokines such as IL-4, IL-13 and TNF-α can increase endothelial permeability, others such as transforming growth factor β1 and IL-10 can reverse the altered permeability[89]. In

addition to their effect on intestinal permeability, pH and motility, inflammatory mediators can alter intestinal drug transporter expression, important proteins responsible for facilitating or antagonizing drug absorption (Section 1.3.1.2). Gastrointestinal availability also depends on the extent to which the drug is metabolized before absorption by the intestinal drug metabolizing enzymes that are at the same time affected by inflammation (Section 1.3.1.1.).

Once the drug reaches the general circulation, either directly after intravenous administration or after absorption from administration sites, it distributes into tissues. Drug distribution depends on many factors such as the physicochemical properties of the drug, the extent of binding to plasma proteins, blood cells and tissue proteins. It has been found that inflammation alters plasma protein levels. After the insult, the body reacts by synthesizing many proteins, collectively called acute phase proteins. These include alpha acid glycoprotein (AAG), CRP, fibrinogen and ceruloplasmin. On the other hand other protein levels are reduced such as plasma albumin and transferrin[90]. These alterations can affect protein binding of drugs. Increased AAG during inflammation reduces the unbound fraction of basic drugs, the fraction that is capable of distribution to tissues and exhibiting the pharmacological action. For example, the unbound fraction of verapamil, propranolol and oxprenolol is reduced in models of inflammation[4, 8, 91].

Inflammation also alters metabolism and excretion of drugs. This has been attributed mainly to inflammation-induced alterations of drug metabolizing enzymes and membrane transporters (to be discussed below in more details).

1.3.1.1.Inflammation and Drug Metabolizing Enzymes

The expression, function and stability of drug metabolizing enzymes are all highly regulated by many exogenous and endogenous factors such as genetic polymorphism, drugs, dietary components, hormones and diseases[92]. Inflammation is one of those conditions that have been found to downregulate drug metabolizing enzymes, notably cytochrome P450s and may have an influence on drug metabolism and toxicity[37]. This effect is of importance if the drug has a narrow therapeutic range or is highly metabolized. It has been reported that inflammation diminishes the clearance of drugs metabolized by CYP450s by 20 to 70 % [93-97]. Inflammation-induced reduction of drug metabolizing enzymes can affect drugs with high and low hepatic extraction ratio. However, the effect is more pronounced in drugs with high hepatic extraction ratio than those with low extraction ratio especially when given by the oral route because of the compromised hepatic first pass metabolism. In addition, reduced drug clearance can also be observed following other routes that bypass the hepatic first pass such as the intravenous route. This is because drug metabolism may be highly compromised and the drug becomes with medium extraction ratio. Therefore, its clearance will be reduced as a result of reduced hepatic intrinsic clearance and increased protein binding[38, 98]. One of the first reported inflammation-drug

interaction cases is the severe theophylline toxicity experienced by children following influenza infection during the influenza epidemic in 1980. The viral infection caused a five-fold increase in theophylline plasma level as a result of infection-induced downregulation of CYP1A2, the main theophylline metabolizing enzyme[99]. It has been reported that inflammation and inflammatory diseases such as rheumatoid arthritis and Crohn's disease reduce the hepatic clearance of many drugs especially for those that are highly cleared by the liver[3, 4, 8, 91, 100-104]. For example, the plasma level of cyclosporine, a CYP3A4 substrate, is elevated after bone marrow transplantation, an observation that is correlated with a pre-elevation of IL-6 plasma level[103]. Also, the plasma level of clozapine, a CYP3A4 and CYP1A2 substrate, is elevated up to the toxic levels in inflammatory diseases[104].

The mechanisms behind inflammation-induced downregulation of cytochrome P450s have been studied extensively[93]. Several cytokines such as IL-1, IL-6, IFN-γ and TNF-α have been implicated. They alter cytochrome P450s at the transcriptional, translational levels through affecting mRNA and protein expression, respectively. Moreover, cytokines can act at the post-translational levels by compromising the enzyme stability or directly inhibiting it[37, 96]. In addition, different cytokines have been found to have different specificities towards different enzymes suggesting the role of the nature of inflammation on the metabolic effect observed[93-97]. To determine the specific cytokine responsible for the observed effect, knock-out animal models have been used. For example,

the downregulating effect of turpentine on cyp1a2, cyp2a5 and cyp3a11 is abolished in IL-6 knock-out mice[105]. On the other hand, IL6 and IL1 β gene deficiency has no influence on lipopolysaccharides-induced downregulation of CYP P450 genes suggesting the redundant effect of various inflammatory mediators[105]. In addition to cytokines, nitric oxide has been implicated to be involved in the downregulation[8, 106] but there are some reports that oppose that notion[107]. In addition, ROS has been found to be involved in the downregulation of CYP P450s[108-110]. Figure 1-1 depicts the possible mechanisms of inflammation-induced downregulation of cytochrome P450s.

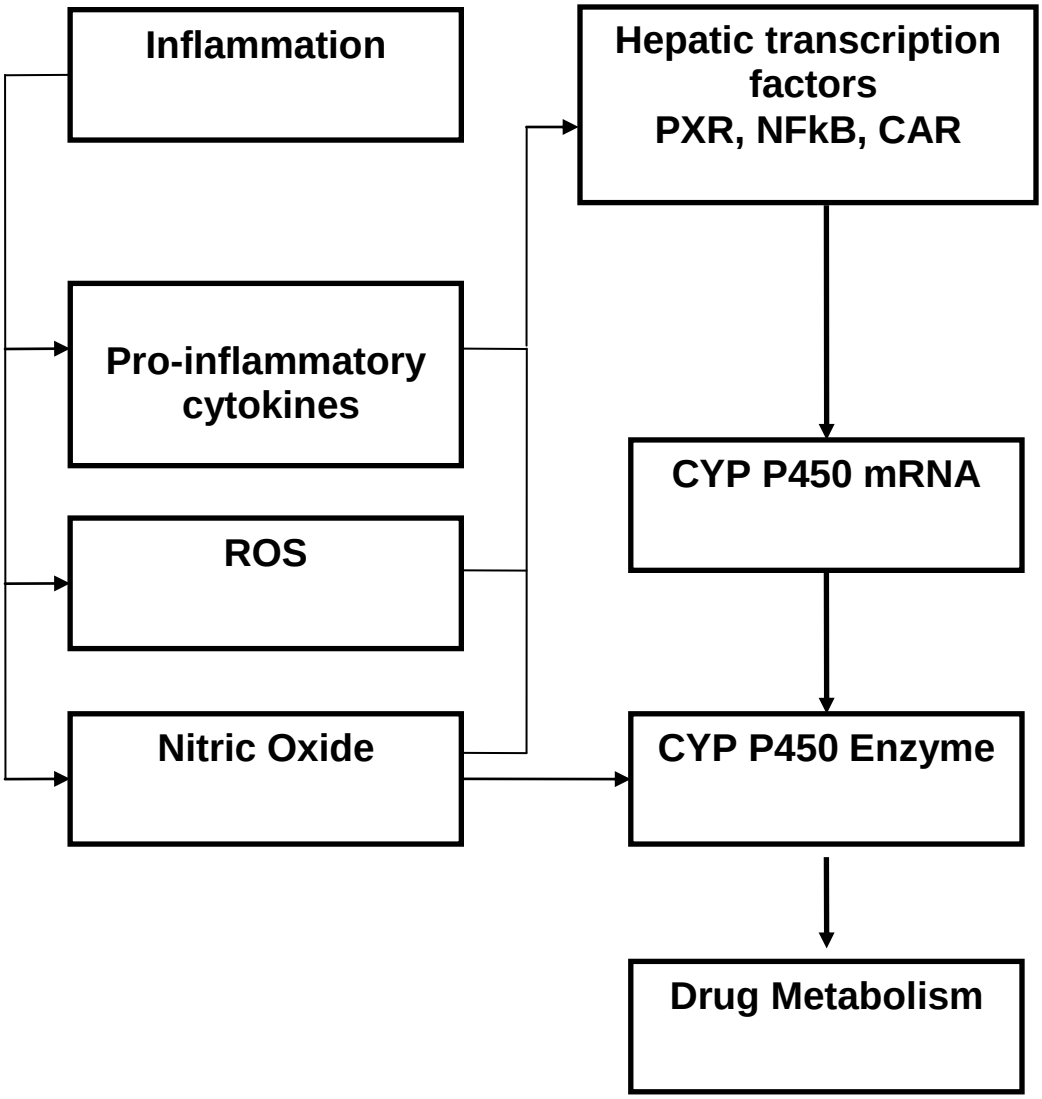


Figure 1-1. Possible mechanisms of inflammation-induced downregulation of cytochrome (CYP) P450s. Cytokines, reactive oxygen species (ROS) and nitric oxide, released as a result of inflammation, inhibit the hepatic transcription factors pregnane X receptor (PXR), constitutive androstane receptor (CAR) and nuclear factor kappa B (NFκB) with subsequent inhibition of the transcription of CYP P450 genes. In addition, nitric oxide directly inhibits CYP enzymes by enzyme destabilization or inhibiting its synthesis.

1.3.1.2.Inflammation and Drug Transporters

The processes of drug absorption, distribution and elimination involve drug crossing through biological membranes. Generally, the process of drug crossing through those membranes may be passive or transporter-mediated[111]. Membrane transporters are proteins involved in the transfer of endogenous compounds such as bile salts, amino acids and sugars, a vital process for cellular nutrition, detoxification and homeostasis. Some of those transporters are capable of facilitating influx and efflux of drugs through biological membranes participating in making up their fate inside the body[112].

Generally, there are two kinds of transporters, influx transporters that uptake the drugs into the cell and efflux transporters that export them out. Uptake transporters include organic anion transporter polypeptides (OATPs), organic cation transporters (OCTs), organic anion transporters (OATs), peptide transporters (PEPT) and organic cation carnitine transporters (OCTN). Efflux transporters include multidrug resistance proteins (MDR) which include P-glycoprotein, multidrug resistance associated proteins (MRP), bile salts export pump (BSEP) and breast cancer resistance protein (BCRP). Efflux transporters use adenosine triphosphate (ATP) as source of energy to allow transport of chemicals[112]. The importance and clinical significance of drug transporters emerged from the understanding of their role in drug pharmacokinetics, disease pathophysiology such as in cystic fibrosis, resistance to anticancer drug therapy and the presence of drug, food and disease-drug interactions at the level of drug

transporters[112]. Table 1-5 depicts a list of selected human drug transporters, their biological localization and their substrates.

Similar to drug metabolizing enzymes, inflammation alters the regulation of drug transporters. Transporter downregulation or upregulation at the transcriptional, translational or post-translational levels is dependent on the transporter itself, the inflammatory model or disease tested, the cytokines involved and the severity of inflammation[113]. This explains the observed discrepancies on transporters' alterations in different inflammatory models. Table 1-6 depicts a summary of studies investigating the effect of inflammatory models and diseases on the expression of drug transporters.

Table 1-5. Selected human drug transporters, their substrates and biological localization. Adapted from references[112-114]

Transporter name (other name)	**Biological localization**	**Drug substrates**
OATP1A2 (SLCO1A2)	Liver, kidney, brain	Indomethcin, fexofenadine
OATP2B1 (SLCO2B1)	Liver, intestine, placenta	Digoxin, penicillin G
OATP1B1 (SLCO1B1)	Liver	Penicillin G, pravastatin, rifampin, methotrexate
OATP1B3 (SLCO1B3)	Liver	Digoxin, methotrexate, rifampin
OAT1 (SLC22A6)	Kidney, brain	Acyclovir, tetracycline
OAT3 (SLC22A8)	Kidney, brain	Methotrexate, salicylate, tetracycline, valacyclovir,
OCT1(SLC22A1)	Liver, intestine, brain	Cimetidine, quinine, quinidine, midazolam, verapamil
MDR1 (P-glycoprotein, ABCB1)	Widely distributed	Digoxin, cyclosporine, protease inhibitors, ketoconazole, verapamil, digoxin, quinidine, , statins, erythromycin, loperamide
BSEP (ABCB11)	Liver	Vinblastine, bile salts,

		tamoxifen
MRP1 (ABCC1)	Widely distributed	Vinca alkaloids, methotrexate
MRP2 (ABCC2)	Liver, kidney, intestine	Cisplatin, methotrexate, ampicillin, ceftriaxone
MRP3 (ABCC3)	Liver, kidney, intestine	Doxorubicin, vincristine, cisplatin
BCRP (ABCG2)	Liver, intestine, placenta	Doxorubicin, methotrexate, mitoxantrone

OATP, organic anion transporter polypeptides; OCT, organic cation transporters; OAT, organic anion transporters; MDR, multidrug resistance proteins; MRP, multidrug resistance associated proteins; BSEP, bile salts export pump; BCRP, breast cancer resistance protein (BCRP).

Table 1-6. The effect of inflammatory models and diseases on the expression of drug transporters.

Transporter	Model/disease	Effect
MDR1 (P-glycoprotein, ABCB1)	Turpentine-induced inflammation in rats[115]	↓ Expression and activity in liver
	Endotoxin-induced inflammation in rats[116-119]	↓ mRNA and protein in liver ↑ mRNA and protein in cerebral microvessels ↓ mRNA and protein in jejunum and kidney
	Endotoxin-induced inflammation in mice[120]	↑ in mRNA and protein in kidney ↓ mRNA and protein in liver ↑ renal clearance of doxorubicin ↓biliary clearance of doxorubicin
	Chronic renal failure in rats[121]	↓ activity in intestine ↔ mRNA and protein in intestine
	dextran sodium sulphate	↓ mRNA and function in large

	(DSS)-induced colitis in mice[122]	intestine
	Ulcerative colitis[123]	↓ mRNA in intestine
	IL-6 in vitro[102, 103]	↓ Expression in liver
	Experimental cholestasis[124]	↔ in intestine
	IFN-γ treatment of Caco-2 cells[125]	↑ mRNA and protein but not activity
	Intrahepatic and	↔ protein in liver
	obstructive cholestasis in rat[126]	↔ protein in liver
	- Endotoxin-induced	↑ protein in liver
	- Ethiny estradiol-induced	
	- Common bile duct ligation	
MRP1	Endotoxin-induced inflammation in rat[127]	↑ mRNA and protein in liver
	IL-6 treated human hepatoma cells in vitro[128]	↑ mRNA

MRP2	Endotoxin-induced inflammation in rat[116, 117, 127]	↓ mRNA and protein in liver
	Intrahepatic and obstructive cholestasis in rat[126]	↓ mRNA and protein in liver
	- Endotoxin-induced	↔ mRNA ↓ protein in liver
	- Ethiny estradiol-induced	↓ mRNA and protein in liver
	- Common bile duct ligation	
	Indomethacin-induced bowel injury in rats[129]	↓ mRNA in liver
MRP3	IL-6 treated human hepatoma cells in vitro[128]	↑ mRNA and activity
OATP1b2	Indomethacin-induced bowel injury in rats[129]	↓ mRNA in liver
BCRP	Ulcerative colitis[123]	↓ mRNA in intestine

OATP, organic anion transporter polypeptides; MDR, multidrug resistance proteins; MRP, multidrug resistance associated proteins; BCRP, breast cancer resistance protein.

The effect of inflammation on the pharmacokinetics of drugs is rather complex. Prediction of those effects depends on the nature and severity of the inflammatory disease. For example the arthritic index in rheumatoid arthritis, a measure for disease severity, was positively correlated with nitric oxide level[3] and the latter in turn is negatively proportional with CYP enzyme levels[8]. In other words, the more severe the inflammation is, the fewer enzymes are available for metabolism. Moreover, prediction of pharmacokinetic changes depends on the characteristics of the drug itself such as drug solubility, fraction metabolized, protein binding, fraction eliminated unchanged and lipophilicity. Knowledge of these properties can allow for prediction of the effects of inflammation. The biopharmaceutics drug disposition classification system (BDDCS)[130], a modification of the biopharmaceutics classification system (BCS)[131], classifies drugs according to their degree of solubility and metabolism and allows prediction of the effect of transporters on each class[132].

1.3.2. Effect of Inflammation on Pharmacodynamics of Drugs

Inflammation can alter drug response. This has been attributed to inflammation-induced alterations of drug pharmacokinetics such as increased protein binding reducing the availability of the biologically active unbound fraction and reducing metabolism thereby increasing drug exposure. However, pharmacokinetics changes can not solely explain inflammation-induced alterations of drug response. Changes at the pharmacodynamic level add to the observed effects of inflammation. For example, despite their increased concentration in inflammatory

diseases and animal models of inflammation, propranolol and verapamil pharmacological potencies are reduced in inflammation[3, 4, 6, 8]. This has been explained by altered receptor functions and abundance[4, 52]. In addition, the pharmacological response to some drugs is altered without any pharmacokinetics changes. For example, while inflammation does not alter sotalol pharmacokinetics, its pharmacological response is reduced[5]. Inflammatory mediators have been found to be implicated in alteration of the receptor function. For example, incubation of rat cardiac myocytes with TNF-α and IL-1β has resulted in reduced L-type calcium channel currents and reduced β-adrenergic functions[110-112]. Inflammation-induced reduction in drug response has been recognized in many inflammatory diseases either directly by in vivo, in vitro and clinical studies or indirectly by affecting disease prognosis and outcome. Increased inflammatory mediator concentrations has been associated with poor disease outcome and/or therapy failure in AIDS, cardiovascular diseases, diabetes, infections, psychiatric illnesses, autoimmune diseases and cancer[38]. Table 1-7 depicts examples of diseases whose outcomes are affected by inflammation.

Table 1-7. Involvement of inflammation in pharmacotherapy outcomes in inflammatory conditions. Adpated from reference[38].

Condition	Cytokine alteration	Therapeutic relevance
Human Immunodeficiency virus (HIV)	↑ TNF-α	Increased resistance to tuberculosis pharmacotherapy Increased antiretroviral therapy failure
Acute Myocardial Infarction	↑ CRP	Increased mortality and poor prognosis
Unstable angina	↑ IL-1ra and IL-6	Complicated hospital stay
Rheumatoid Arthritis	Systemic inflammation	Decreased antiarrhythmic potency of verapamil
Diabetes Mellitus	↑ TNF-α in skeletal muscles	Insulin resistance
Obesity	Low grade systemic inflammation	Reduced verapamil potency
Elderly	Low grade systemic inflammation	Reduced verapamil potency β-blockers inefficiency in elderly
Allergy	↑ IL-5 ↑ IL-6	Steroid resistant asthma Antihistaminic failure in

		urticaria
Cancer	↑ cellular TNF-α	Doxorubicin resistance
	↑ TGF-β	Anticancer failure in prostate carcinoma
	↑ TGF-β2	Tamoxifen resistant breast cancer
	Th1 polarization	Cyclophospahamide resistance
Infection	↑ plasma TNF-α	Poor tuberculosis prognosis
	↑ proinflammatory mediators	Steroid failure in leprosys patiants
Pain	Chemokine receptor activation	Reuced opioid potency
Psychiatric illnesses	↑ IL-6	Therpaeuic failure in depression and psychosis

TNF, tumor necrosis factor; CRP, C-reactive protein; IL, interleukin; TGF, transforming growth factor.

1.3.2.1.Inflammation and L-type Calcium Channels

Voltage-gated calcium channels are one of the most important channels responsible for coupling the change in membrane potential to intracellular signaling. Voltage-gated calcium channels undergo voltage dependent activation, a finely regulated process by a large number of extracellular and intracellular mechanisms[133]. They differ from voltage-gated potassium and sodium channels in that not only are they involved in the change in membrane potential but also in many physiological processes mediated by calcium such as contraction, secretion and gene expression[133].

Voltage-gated calcium channels are classified according to their electric characteristics into low-voltage and high voltage activated channels. Low-voltage channels, activated by low depolarizing current and inactivated rapidly, include T-type calcium channels which are abundant in sinoatrial pacemaker node as well as purkinje fibers[133, 134]. High-voltage activated channels need high voltage for depolarization but they undergo variable inactivating times (intermediate to slow)[135]. The most important of this group is L-type calcium channels; others include P/Q, N and R types[136]. Structurally, voltage-gated calcium channels consist of a complex of the pore forming functional α_1 subunit and other modulating subunits, α_2 δ, β, and γ[134, 137, 138]. $\alpha 2$ δ is actually two subunits expressed from the same gene and linked by a disulphide linkage[139]. Alpha 1 (α_1) subunits share a common structure consisting of four transmembrane domains (I, II, III and IV). Each domain is composed of 6 putative transmembrane segments

(S1 to S6) and two short segments SS1 and SS2. S4 of each domain confers the voltage sensing sequence for channel activation. S5 and S6 segments are thought to line the channel, a structural feature shown by hydropathy plots and site directed mutagenesis[137]. Calcium channels are classified according to their electric properties and their α_1 subtype into three families, Ca_v1, 2 and 3[134, 136]. Voltage-gated calcium channels are expressed in many tissues especially excitable cells such as skeletal and smooth muscle cells and neurons. Tissues exhibit specific expression of calcium channel subunits, for example while α_{1C} is expressed in cardiac and smooth muscles and neuronal tissues, α_{1D} is expressed in endocrine and neuronal tissues[134, 136, 137]. Table 1-8 depicts the classification and biological distribution of voltage-gated calcium channels.

Table 1-8. Classification and biological distribution of voltage-gated calcium channels. Adapted from references[136].

Channel Family	Channel Name	old name	Conventional Name	Biological Distribution
Ca_V1	Ca_v 1.1	α_{1S}	L-type	Skeletal muscles
	Ca_v 1.2	α_{1C}		Cardiac muscles, endocrine cells, neurons
	Ca_v 1.3	α_{1D}		Endocrine cells, neurons, pacemaker cells, atrial myocytes
	Ca_v 1.4	α_{1F}		Retina, spinal cord, adrenal glands, mast cells
Ca_V2	Ca_v 2.1	α_{1A}	P/Q-type	Neurons
	Ca_v 2.2	α_{1B}	N-type	Neurons
	Ca_v 2.3	α_{1E}	R-type	Neurons
Ca_V3	Ca_v 3.1	α_{1G}	T-type	Neurons, myocytes
	Ca_v 3.2	α_{1H}		Neurons, myocytes
	Ca_v 3.3	α_{1I}		Neurons

L-type calcium channels are essential for coupling the membrane excitation to muscle contraction. Upon channel activation and calcium influx, calcium induces more calcium release from the intracellular stores in the sarcoplasmic reticulum with subsequent muscle contraction. Following muscle contraction, off reactions occur helping to restore basal calcium concentration and muscle relaxation. This involves reuptake by sarcoplasmic reticulum and mitochondria and cellular efflux via sodium/calcium exchangers and plasma membrane calcium ATPase. In addition, calcium entry through those channels is responsible for neurotransmitter release, synaptic plasticity and gene expression. Increased influx of calcium is also responsible for phase 2 plateau in myocytes action potential and atrioventricular node (AV node) conduction[134, 136]. Hence, blocking L-type calcium channels can delay AV conduction and prolong PR-interval in electrocardiography (ECG). Functional modulation of calcium channels depends on the balance among kinases such as protein kinase A (PKA) and protein kinase C (PKC) and phosphatases[138, 140]. G-protein-coupled receptors have an important influence on the expression and function of L-type calcium channels (Ca_v1). Activation of β-adrenergic receptors (G-protein-coupled) enhances calcium current through channel phosphorylation, a process mediated by PKA activated downstream increased cAMP level[141]. Stimulation of M3 muscarinic receptors results in increased calcium current due to increased phosphorylation mediated by PKC, activated downstream phospholipase C activation. On the other hand, activation of M2 muscarinic receptors in cardiac

muscles results in reduced calcium current due to reduced channel phosphorylation resulting from cAMP inhibition[138].

Altered L-type calcium channel expression and function have been implicated in disease pathophysiology and symptomatology such as in hyperthyroidism and atrial fibrillation. Hyperthyroidism is associated with exertional dyspnea and symptoms of heart failure. This has been attributed to thyroid hormone-induced downregulation of L-type calcium channels in the myocardium[142]. In addition to hyperthyroidism, altered L-type calcium channel functions play a role in the pathogenesis of atrial fibrillation. It has been found that atrial fibrillation is associated with reduced calcium current with subsequent shortening the atrial effective refractory period and hence reducing action potential duration[134]. Reduced calcium channel current in atrial fibrillation has been explained by reduced $Ca_v1.2$ expression[143, 144], reduced β-subunit expression[145] and increased protein phosphatase activity[140].

Inflammation has been found to alter L-type calcium channel expression and function. L-type calcium channels are downregulated in different models of inflammation[4, 132]. This has been attributed to inflammatory mediators and other acute phase reaction products. For example, in vitro incubation of TNF-α and IL-1β with rat cardiac myocytes resulted in reduced L-type calcium channel currents[146, 147]. Binding of inflammatory mediators to their receptors and release of ROS activates the intracellular transcription protein nuclear factor kappa B

(NFκB)[148]. The latter, in turn, is translocated to the nucleus to act on specific response elements on DNA modulating the expression of some proteins[149]. One of those affected proteins is the L-type calcium channel[150]. In addition to calcium channel downregulation, inflammation alters the downstream signaling mechanisms affecting calcium channels functions. It has been found that acetylcholine-induced calcium current augmentation is compromised in inflamed dogs. This has been attributed to alteration of the second messenger system coupling muscarinic receptor activation to L-type calcium channels such as reduced expression of G-q11 protein and altered PKC[136].

Inflammation-induced downregulation of L-type calcium channels appears to have subsequent pathophysiological and therapeutic consequences. Chronic inflammatory bowel diseases and animal models of colonic inflammation are characterized by reduced colonic contractions and muscle tone[151] as a result of $Ca_v1.2$ downregulation in colon[152]. Heart failure and septic shock-induced atrial fibrillation are other examples of diseases associated with inflammation-induced calcium channel alterations[12, 134, 153-155]. Pharmacodynamic response to calcium channel blockers is reduced in inflammation models and in obesity[1, 2, 4], observations that can adversely affect disease outcomes.

1.3.3. Example of Inflammation-Drug Interaction: Verapamil

The critical role of L-type calcium channels in the cardiovascular system renders them important therapeutic targets for treatment of cardiovascular diseases by the use of L-type calcium channel antagonists. This group includes three main classes: phenylalkylamines (e.g. verapamil), benzothiazepines (e.g. diltiazem) and dihydropyridines (DHP) (e.g. nifedipine and amlodipine). They interact with their receptor sites on the S6 segments of the transmembrane domains III and IV of the functional α1 subunit (Ca_v1). These sites are responsible for the high affinity and specificity of these drugs[135, 137]. Calcium channel blockers relax arterial smooth muscles by inhibiting calcium entry into the cells and inhibiting excitation-contraction coupling[39]. Sinoatrial (SA) node excitability and AV nodal conduction depend mainly on calcium entry. Hence, CCBs which delay the recovery of slow calcium channels will have an effect on SA and AV nodal excitability and conduction[156]. While, non-dihydropyridines, diltiazem and verapamil, are able to do so, DHPs at clinical doses can not affect SA and AV nodes. In contrast, dihydropyridines have the greatest peripheral vasodilatation effects and negligible action on the heart. As a result, feedback reflex tachycardia may occur[39]. Calcium channel blockers are indicated in the treatment of hypertension, angina (variant, exertional, unstable), AMI (verapamil and diltiazem in post-AMI by reducing the incidence of re-infarction), cardiac arrhythmias (verapamil and diltiazem only), migraine prophylaxis (verapamil), heart failure (amlodipine), cerebral vasospasm (nimodipine) and Raynaud's disease (DHPs and diltiazem)[39].

Verapamil [2, 8-bis-(3, 4-dimethoxyphenyl)-6-methyl-2-isopropyl-6-azaoctanitrile] is a synthetic papaverine derivative that was first recognized and described in 1962 by Albrecht Fleckenstein. Despite being a phenylalkylamine calcium channel blocker, verapamil was formerly considered as a β-adrenoceptor blocker. Afterward, it has been found that its pharmacological actions are due to reduction of calcium entry into the cell and prevention of excitation-contraction coupling[157].

Verapamil is well absorbed from the gastrointestinal tract[158], however; it undergoes extensive hepatic first pass metabolism significantly reducing its oral bioavailability. In addition, gut metabolism has been found to play a role in reducing verapamil bioavailability[159, 160]. Verapamil oral bioavailability has been reported to range from 18 to 25%[161-163]. It is highly metabolized in the body with very little fraction (<5%) excreted unchanged in urine[164]. Being a chiral compound, verapamil exists in a racemic mixture of S and R enantiomers. In human, the body exhibits stereoselective metabolism in a way that the S-enantiomer is rapidly cleared than the R-enantiomer[165]. Verapamil is highly bound to plasma proteins, AAG and albumin. Stereoselective protein binding between R-verapamil (94% bound) and S-verapamil (88% bound) also exists[165]. Stereoselective metabolism and protein binding explain the observed higher R-verapamil plasma concentration in man[3, 162, 165].

Following intravenous administration, verapamil plasma concentration vs time profile has been best described by a two-compartment model with a distribution half-life that ranges from 5 to 8.5 min and terminal half-life between 2 to 8 hrs[161-163, 165]. The wide range of verapamil half-lives can be attributed to age and gender differences observed among healthy volunteers enrolled in pharmacokinetic studies[162]. The effect of increased clearance balances the effect of increased volume of distribution on plasma half-life of S-verapamil. This explains why S- and R-verapamil have half-lives similar to that observed in racemic verapamil[165].

Verapamil pharmacokinetics have been studied in several animal species such as dogs[166], rabbits[167] and rats[4, 8, 168]. The stereoselective metabolism and protein binding of verapamil enantiomers are species specific. While dogs exhibit much higher R-verapamil concentrations than S-verapamil similar to humans[166], verapamil disposition in rabbits is not stereoselective[167]. On the other hand, stereoselective metabolism and protein binding of verapamil in rats are reversed as compared to humans[169]. In other words, the systemic clearance and volume of distribution of R-verapamil are much higher than S-verapamil. Verapamil terminal half life in rats ranges from 1.7 to 2.4 hrs[168].

Considering the chemical structure of verapamil (Figure 1-2), there are several possible metabolites. However, only three main metabolites have been observed, nor-verapamil, D-702 and D-617. Nor-verapamil and D-617 are

produced by N-dealkylation, while D-702 is produced by O-demthylation. These metabolites can be further biotransformed to PR-25, PR-22 and D-620[164, 170]. Figure 1-2 depicts the major routes of verapamil metabolism. Nor-verapamil is the most important metabolite since it is pharmacologically active but with very low potency[171]. Cytochrome P450 enzymes are responsible for the oxidative metabolism of verapamil. While CYP3A4, CYP3A5 and CYP1A2 isozymes are involved in the N-dealkylation and N-demethylation of S and R verapamil enantiomers[170, 171], CYP2C8 isozyme is involved in the O-demethylation pathway[170, 172]. Thus, any factor that can influence the transcription, translation and/or activity of these enzymes can influence verapamil pharmacokinetics in the body.

When taken in therapeutic doses, verapamil delays AV conduction leading to PR-interval prolongation even after small doses[161], this action is known as negative dromotropism. The degree of PR interval prolongation is plasma concentration dependent[161, 173]. In concentration-response curves, racemic verapamil concentration vs. percentage of PR-interval prolongation, the slope following iv dosing is steeper than oral dosing. In other words, plasma concentrations required to achieve the same response after oral dosing are 2 to 3 times higher than those following iv dosing in man[163]. This is because S-verapamil, which is 10 to 20 times more dromotropically potent than R-verapamil[174, 175], undergoes extensive first pass metabolism[165].

Verapamil metabolism is altered in inflammatory conditions. Verapamil key metabolic enzymes CYP3A, CYP1A2 and CYP2C are downregulated in inflammation[8, 37, 117, 176]. It has been found that the hepatic CYP P450 content and the subfamilies CYP3A and CYP1A are downregulated in pre-adjuvant arthritis rats and negatively correlated serum nitrite and TNF-α to varying degrees[8]. As a result, plasma concentrations of both verapamil enantiomers were elevated in animal models of inflammation (Figure 1-3)[4, 8] and in rheumatoid arthritis patients (Figure 1-4)[3]. In addition to reduced clearance, increased plasma verapamil concentration observed in inflammatory diseases is a function of increased plasma protein binding to AAG[8], an acute phase protein that is elevated in patients with inflammatory disorders[177]. P-glycoprotein is another factor in the inflammation-pharmacokinetic relationships that should be considered. Being an active efflux pump, it plays an important role in the exsorption and elimination of drugs[178]. It has been found that P-glycoprotein expression and activity are reduced in animal models of inflammation[118, 179]. Being a P-glycoprotein substrate[178], verapamil enhanced oral bioavailability may be caused, at least in part, due to reduced P-gp expression.

In inflammation, elevated plasma concentration of verapamil was expected to increase verapamil pharmacological actions (drug-induced PR-interval prolongation) and causing even toxicity (heart block). In contrast, verapamil potency has been found to be reduced in both human[3] and animal models of inflammation[4]. Reduced verapamil concentration at the site of action as

a result of inflammation-induced increase in plasma protein binding is an explanation put forward to explain the reduced verapamil response[3]. However several findings have ruled out the role of protein binding. First, verapamil negative dromotropic effect is reduced in elderly with minimal changes in protein binding[173]. Second, the free unbound fraction of the more potent S-verapamil is not altered in rheumatoid arthritis patients[3]. Third, the less potent nor-verapamil[180] can displace verapamil from protein binding sites[171] increasing the free fraction of verapamil. Last, the pharmacological response to nifedipine is reduced without any pharmacokinetics alteration suggesting that the observed effect of inflammation is at the pharmacodynamic level[4]. Radiolabeled ligand binding studies using H^3-nitrendipine have shown that inflammation downregulates L-type calcium channel maximum binding capacity (Bmax) in rat cardiac cell membrane preparations without affecting the dissociation constant (Km), a measure of receptor affinity[4]. Therefore, the reduced response of calcium channel blockers is not due to competitive inhibition otherwise the concentration versus Bmax curve should shift to the right with change in Km[181]. Moreover, reduced Bmax can be explained by either non-competitive inhibition or reduced receptor density[181].

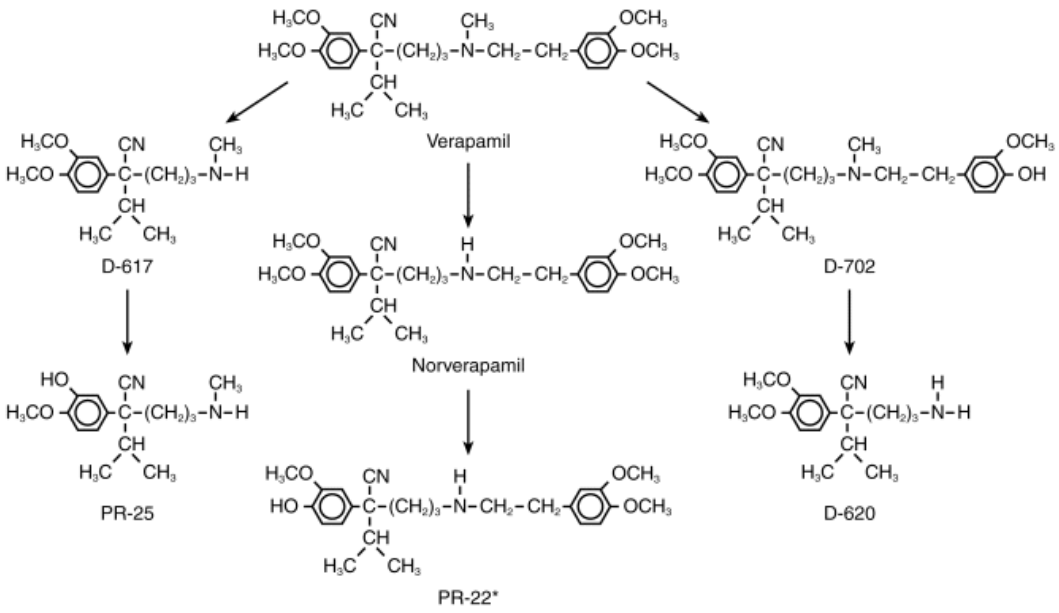


Figure 1-2. Pathways of verapamil metabolism in man[170].

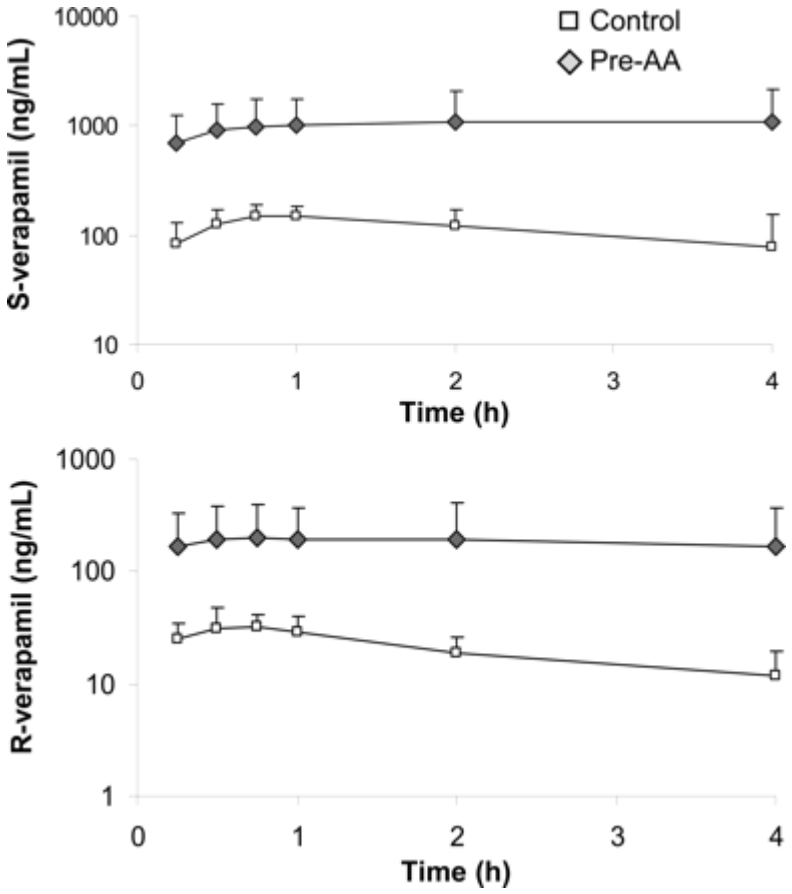


Figure 1-3 Effect of inflammation on plasma concentrations of verapamil enantiomers in pre-adjuvant arthritis (Pre-AA) rats as compared to control[8].

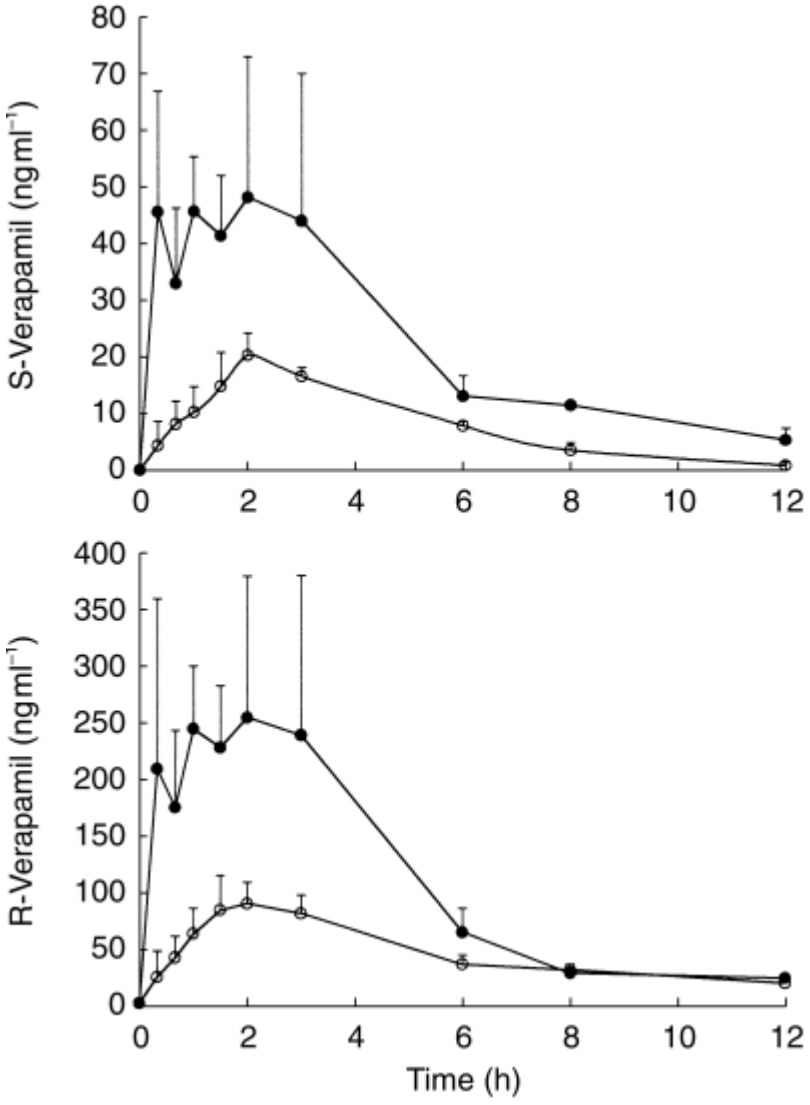


Figure 1-4 Effect of inflammation on plasma concentrations of verapamil enantiomers in rheumatoid arthritis patients (●) compared to healthy ones (○)[3]

1.3.4. Control of Inflammation

Inflammation plays an important role in the pathogenesis and progression of many diseases and conditions. In addition, it can complicate the disease course by altering pharmacotherapy outcomes through affecting the pharmacokinetics and pharmacodynamics of drugs. Therefore, it is desirable to control inflammation to stop the progression of the disease, to relief the symptoms as well as to restore altered drug actions and disposition to improve therapeutic outcomes. Generally, there are two modalities to control inflammation. The first modality is to treat the disease itself, if curable, such as treating infections with antimicrobial agents and losing weight in obesity. The second modality is the use of anti-inflammatory agents. Anti-inflammatory agents are heterogeneous groups of drugs that act by different mechanisms of actions on different inflammatory pathways. Table1-9 depicts a list of the anti-inflammatory agents discovered to date. Moreover, other therapeutic agents have been found to possess anti-inflammatory properties in addition to their main pharmacological actions such as statins and angiotensin-interrupting agents (Section 1.3.4.1.). Statins are inhibitors of the enzyme hydroxymethylglutaryl coA reductase, a key enzyme involved in cholesterol synthesis[39]. They are indicated to control hypercholesterolemia, an important cardiovascular risk factor[48]; however, the beneficial actions of statins extend beyond their cholesterol lowering properties. Statins possess antioxidant and anti-inflammatory actions through inhibiting pro-inflammatory mediators' expression, immune cell migration, NFκB activation and endothelial adhesion[182, 183].

The benefits of the use of anti-inflammatory agents in cardiovascular diseases such as heart failure and acute myocardial infarction have been studied. While some drugs have been found to improve cardiovascular long-term outcomes and reduced cardiovascular complications such as angiotensin interrupting agents and statins[184-187], others failed to provide sufficient benefit. Anti-TNF therapy by infliximab has been found to reduce IL-6 and CRP concentrations in patients with heart failure, however neither infliximab nor etanercept were able to cause any significant benefit[188, 189]. It is not clear whether the use of corticosteroids such as methylprednisolone in myocardial infarction patients is beneficial. Some studies have found that it can limit the infarct size by suppressing inflammation others did not support that notion[190]. Few studies have determined the benefit of inflammation control by statins, NSIADs and infliximab on inflammation-induced alterations in pharmacokinetics and/or pharmacodynamics of drugs. Statin therapy has been found to restore the reduced pharmacological actions of propranolol without altering its reduced clearance in pre-adjuvant arthritis rats[52]. On the other hand, treatment of adjuvant arthritic rats with ketoprofen reduced propranolol exposure (area under the concentration-time curve) as a result of reducing disease severity[191]. Disease control by infliximab restored the altered verapamil pharmacokinetics in rheumatoid arthritis patients and partially restored inflammation-induced reduction of hepatic cytochrome P450 content in pre-adjuvant arthritis rats without normalizing verapamil pharmacokinetics[192, 193]. The latter has been explained by the failure of infliximab to normalize inflammation-induced protein binding in rats[192].

Table 1-9 List of anti-inflammatory agents. From references[18, 39].

Anti-inflammatory agent	Description
Non-steroidal anti-inflammatory drugs (NSAIDs)	
Non-Selective NSAIDs	They inhibit prostaglandins synthesis by inhibiting both COX-1 and COX-2 enzymes; they control inflammation and associated pain; however, they do not stop disease progression.
Selective COX-2 inhibitors	They selectively inhibit COX-2 enzyme, as a result they have minimal gastrointestinal adverse effects; their use has been associated with increased cardiovascular events.
Corticosteroids	
	They are a group of drugs that have myriad pharmacological actions and a many therapeutic indications; they inhibit the synthesis of the pro-inflammatory prostaglandins and leukotrienes; they suppress immune cells, inhibit Th1 cells and decrease the production of pro-inflammatory mediators; they are indicated in autoimmune diseases, asthma, organ transplants, renal diseases and most of diseases complicated by inflammation; because of their serious

	adverse effects they are indicated only for short-term use.
Disease modifying antirheumatic drugs (DMARDs)	
Sulfasalazine, methotrexate, penicillamine, hydroxychloroquine, leflunomide and gold compounds	They are a group of drugs that are indicated in autoimmune diseases such as rheumatoid arthritis (RA) to stop progression of the disease rather than just symptomatic treatment; DMARDs are now indicated in the early course of the disease rather than a second line of therapy; because of the risk of serious adverse effects, gold compounds should only be reserved for severe non-responsive cases of RA.
Immunosuppressives	
6-mercaptopurine and azathioprine	They interferes with DNA synthesis and suppress lymphocyte function; they are used in RA and organ transplant.
Methotrexate	It blocks DNA synthesis by inhibiting the enzyme dihydrofolate reductase; it is used in autoimmune diseases and cancer.
Cyclosporine and tacrolimus	They inhibit calcineurin phophatase with subsequent inhibition of IL-2 expression, an important T-lymphocyte proliferators; they are indicated in organ transplants, RA and psoriasis; their main adverse effect is nephrotoxicity.

Alemtuzumab	It is a humanized monoclonal antibody against CD52, a glycoprotein expressed on the surface of immune cells resulting in cell apoptosis; it is used in leukemia and has potential use in organ transplantation.
Antithymocyte globulins and antilymphocyte globulin	They are antibodies against T-lymphocytes that act by direct cellular toxicity; they are used to induce immunosuppression and in acute organ transplant rejection.
Muromonab CD3	It is a monoclonal antibody against CD3 on T-cell receptor; it inhibits the antigen recognizing capacity of T-lymphocytes; it is used in acute organ transplant rejection; its serious adverse reaction (cytokine release syndrome) limits its use.
Mycophenolate mofetil	It is an inhibitor of T-lympocyte proliferation; it is indicated in organ transplants.
Sirolimus	It is an inhibitor of IL-2-induced lymphocyte proliferation. It is indicated in organ transplants.
Anti-TNF therapy	
Infliximab	It is a chimeric anti-TNF-α monoclonal antibody; it is used to control disease progression in RA and inflammatory bowel diseases.
Etanercept	It is a human recombinant TNF-α receptor bound to IgG; it binds to TNF-α inhibiting its actions; it is

	indicated in RA.
Adalimumab	It is a humanized anti-TNF-α antibody; it is used in rheumatoid arthritis.
Anti-IL-2 receptor antibodies	
Basiliximab and daclizumab	They inhibit IL-2 receptors on the surface of activated T-lymphocytes inhibiting the proliferative action of IL-2; they are used in prophylaxis of transplant rejection.
IL-1 receptor antagonist	
Anakinra	It is a humanized IL-1ra; it is used in rheumatoid arthritis to replenish the pathological alterations in IL-1/IL-1ra balance; in rheumatoid arthritis, it delays the rate of joint erosion however it is less effective than anti-TNF therapy[194].
Leukotriene receptor antagonists	
Montelukast and Zafirlukast	They are competitive cysteinyl leukotrienes receptor 1 (cys-LT1) antagonists; leukotrienes are 1000 times more potent than histamine in causing bronchoconstriction through cys-LT1 receptor; therefore inhibiting cyc-LT1 is favorable in asthma.

Anti-IgE therapy	
Omalizumab	It is a humanized monoclonal antibody; it binds IgE preventing IgE-induced mast cell and basophils activation in allergic reactions; it is indicated in allergic reactions and moderate to severe asthma.
Mast cell stabilizers	
Cromolyn sodium and Nedocromil sodium	They inhibit bronchial mast cell degranulation and inhibit leukocyte migration in pulmonary airways; they are indicated in mild to moderate asthmatic attacks.

1.3.4.1. Angiotensin II interruption

The discovery of the renin-angiotensin system (RAS) dates back from 1898 when Tiegerstedt and Bergman discovered a pressor chemical extracted from the kidney called renin[195]. This was followed by a sequence of discoveries that shaped the current knowledge of the RAS system (Figure 1-5).

The key peptide in the RAS is the octapeptide angiotensin II (AGII). It is produced by a two-step proteolytic reaction. First renin, an enzyme released from the juxtaglomerular cells in the kidney converts angiotensinogen into angiotensin 1 (AGI). Then, AGII is produced from AGI by the action of angiotensin-converting enzyme (ACE), a dipeptidyl carboxypeptidase, which cleaves a dipetide from AGI. Renin secretion from the kidney is controlled by two pathways, local and neurogenic. Local control of renin release is mediated by the macula densa and the baroreceptors in the kidney. Renin release is stimulated by the macula densa as a result of reduced sodium reabsorption and/or by the activated baroreceptors in the kidney in response to reduced blood pressure in the preglomerular vessels. On the other hand, increased sodium reabsorption or blood pressure inhibits renin release. The neurogenic control of renin release involves activation of β1-adrenoceptors on juxtaglomerular cells as a result of norepinephrine release from noradrenergic nerves in the kidney[39].

The discovery of the ACE homolog, ACE-2, added to the complexity of the RAS[196, 197]. ACE-2 catalyzes different biological reactions as compared to

ACE[198]. ACE-2 converts angiotensin I to angiotensin (1-9) which is then converted to angiotensin (1-7). The latter is also produced from the action of ACE-2 on AG II. Angiotensin (1-9) produced by ACE-2 has no known biological activity; however, angiotensin (1-7) is a vasodilator[39, 198]. While ACE-2 knockout mice have reduced cardiac contractility and ventricular enlargement, ACE and ACE-2 double knockout have normal cardiac functions[199]. This suggests the cardioprotective function of ACE-2 and the existence of a balance between ACE and ACE2 to regulate blood pressure, fluid and electrolyte homeostasis.

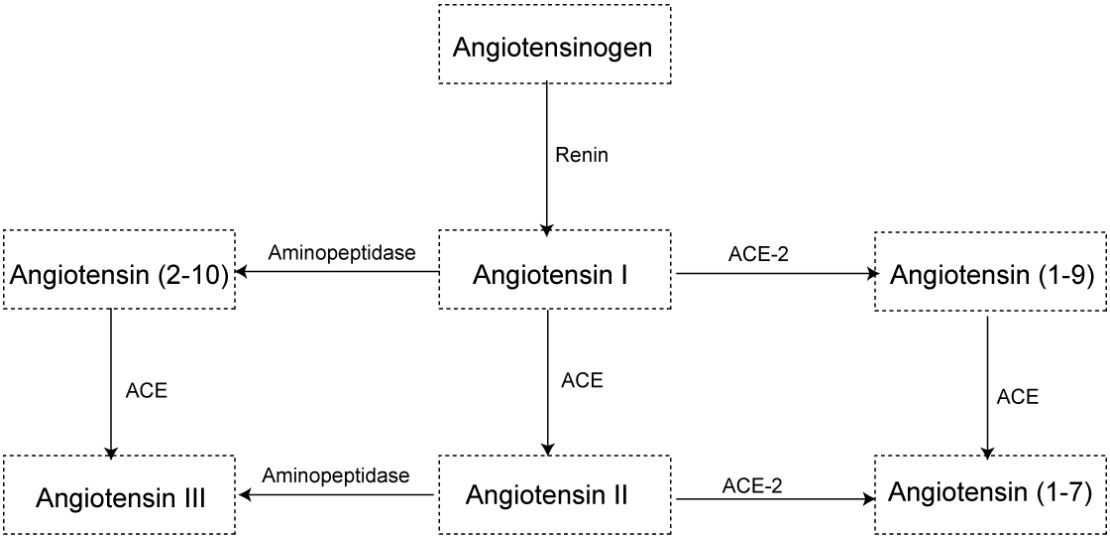


Figure 1-5. Overview of the Renin-Angiotensin System (RAS). ACE, angiotensin converting enzyme; ACE-2, angiotensin converting enzyme 2.

As depicted in Figure 1-5, The RAS system results in production of several polypeptides that have different biological activities[39]:

- **Angiotensin I** – It has very low potency (<1%) as compared to AGII
- **Angiotensin (1-7)** – It is a vasodilator, natriuretic, stimultor of prostaglandin production and inhibitor of vascular smooth muscles proliferation. It does not possess the actions of AGII.
- **Angiotensin II** – It is the most potent peptide. Angiotensin II acts on two G protein–coupled receptors, named AT1 and AT2[200, 201]. However, most of the actions of AGII are mediated by AT1 receptor. Angiotensin II is a powerful direct vasoconstrictor which is 40-fold more potent than norepinephrine. In addition, it enhances the sympathetic neurotransmission and stimulates norepinephrine release from the adrenal glands adding to its vasopressor effect in elevating blood pressure. It also potentiates sodium and water retention as well as potassium excretion in the kidney by direct and indirect effects. It directly stimulates sodium reabsorption at the distal tubules. The indirect effect is through stimulation of aldosterone release from the adrenal cortex. AGII has been found to be a pro-inflammatory mediator implicated in the pathogenesis of inflammatory diseases[202].
- **Angiotensin (2-8)** – It is also called **Angiotensin III**. It has actions similar to AGII; however, it is less (<26%) potent.

Angiotensin interrupting agents (AGIs) include angiotensin converting enzyme inhibitors (ACEIs) and angiotensin receptor blockers (ARBs). ACEIs exert their pharmacological actions by inhibition of ACE enzyme, thus preventing AGII synthesis. They include captopril, ramipril, enalapril, benazepril, lisinopril, quinapril, fosinopril, trandolapril and perindopril. The members of the group differ in their potency and pharmacokinetics; however, there is no preference in their selection as they all have the same pharmacological properties, the same indications and adverse effect profile. ARBs exert their pharmacological actions by competitive inhibition of AT1 receptors and subsequent inhibition of AGII actions (vasoconstriction, aldosterone and catecholamines secretion). They include telmisartan, irbesartan, losartan, olmesartan, valsartan and candesartan. AGIs are indicated in hypertension, heart failure, left ventricular systolic dysfunction, diabetic nephropathy, stroke prevention and reducing mortality following acute myocardial infarction[39].

Both ACEIs and ARBs inhibit the renin angiotensin system leading to similar pharmacological effects; however, because of their different mechanism of action they differ in several characteristics. First, bradykinin level increases under ACEIs therapy. This is because ACEIs inhibit ACE enzyme that breaks down bradykinin. This effect is responsible for the dry cough found in some patients treated by ACEIs. Second, ARBs mainly inhibit AT1 receptors allowing AGII to activate AT2 receptors which has been thought to have effects opposite to AT1 activation[203]. Third, ARBs are more efficient inhibitors of AGII than ACEIs

because the latter do not inhibit the alternative pathways involved in AGII production. Last, ARBs have the capacity to increase the level of the vasodilator AG(1-7) than ACEIs because ACE has been found to be involved in its disposition (Figure 1-5)[39]. Many clinical studies have shown the beneficial effects of inhibiting the actions of angiotensin II in cardiovascular diseases[204, 205]. Their beneficial effects are not only due to their antihypertensive effects but also due to their anti-inflammatory properties.

In addition to its vasopressor functions, angiotensin II has pro-inflammatory properties. It has been found that RAS is activated in patients with inflammatory diseases such as rheumatoid arthritis[206-208]. Angiotensin II has been implicated in the pathogenesis of atherosclerosis, cardiac hypertrophy and heart failure following myocardial infarction[209]. AGII stimulates production of reactive oxygen species, activates NFκB and downregulates peroxisome proliferator–activated receptors (PPARs)[210-212]. Angiotensin II activates NADPH-oxidase enzyme which is responsible for the production of reactive oxygen species[210]. The latter is responsible for endothelial dysfunction and lipid oxidation, two processes involved in atherosclerosis[211]. Activated NADPH-oxidase has been found to complicate hypertension, heart failure and diabetes[213, 214]. In addition, NFκB, an important transcription factor implicated in inflammatory responses, is activated by AGII[148]. Following nuclear translocation, NFκB stimulates the transcription of several proteins including pro-inflammatory mediators such as IL-6, TNF-a and MCP-1[202]. AGII-induced PPARs inhibition leads alteration of insulin signaling

and subsequent insulin resistance[215]. Because of its pro-inflammatory properties, targeting angiotensin II inhibition is desirable. AGIs treatment in a rabbit model of myocardial infarction resulted in reversal of the observed reduced β-adrenergic response[216]. In addition, several studies have found that treatment with ACEIs or ARBs results in reduction of TNF-α, IL-1, IL-6 and MCP-1 concentrations in patients with cardiovascular diseases and those undergoing bypass surgery[217-220]. Di Napoli and Papa have found that including ACEIs therapy in long term stroke patients results in significant reduction of CRP concentrations[184]. Unlike L-type calcium channel and β-adrenergic blockers, inflammatory conditions did not reduce the response to the ARBs, valsartan[221] and losartan[222]. Indeed, for valsartan, a trend toward increased potency has been observed[221].

1.4. Thesis Rationale, Objectives and Hypotheses

1.4.1. Thesis Rationale

- Inflammation complicates many diseases and conditions including rheumatoid arthritis, acute myocardial infarction and obesity.
- Inflammation alters the pharmacokinetics and pharmacodynamics of many drugs in humans and animals. This may explain, at least in part, the poor outcome observed in inflammatory diseases.
- Verapamil is an example of those drugs that have altered pharmacokinetics and pharmacodynamics in inflammation. It has been found that the plasma verapamil concentration is significantly elevated in rheumatoid arthritis

patients and despite that increased level, verapamil-induced ECG changes (PR-interval prolongation) are reduced.

- Valsartan, an angiotensin II receptor blocker, has been found to have anti-inflammatory properties and not affected by inflammation, reversed the diminished pharmacodynamics of verapamil in pre-adjuvant arthritis model.

1.4.2. Thesis Hypothesis

- The diminished response to verapamil caused by inflammation is due to reduced calcium channel target protein.
- The diminished potency of verapamil in arthritis is reversed by angiotensin II inhibition by preventing the downregulation of the target protein (L-type calcium channels)
- Unlike pharmacodynamic changes, valsartan treatment does not reverse inflammation-induced changes in verapamil pharmacokinetics.
- Similar to arthritis verapamil potency is reduced in AMI rats.
- Obese children are less responsive to calcium channel blockers compared to non-obese.
- Obese children are equally responsive to angiotensin interrupting agents compared to non-obese.
- The housekeeping gene 18s rRNA is the least affected gene by inflammation.
- Inflammation alters drug targets and transporters, possible mechanisms of inflammation-drug interaction.

1.4.3. Thesis Objectives

- To determine the effect of valsartan treatment on the altered verapamil pharmacokinetics following oral administration in pre-adjuvant arthritis rats, a model of human rheumatoid arthritis.
- To study the mechanism behind valsartan interaction at the pharmacodynamic level using radioligand binding and Western blot.
- To investigate whether verapamil pharmacodynamic effects (PR-interval prolongation and heart rate reduction) are altered in isoproterenol-induced acute myocardial injury rat model (AMI) similar to what has been found in arthritis models.
- To determine the effect of obesity on the antihypertensive effects of calcium channel blockers and angiotensin interrupting agents (angiotensin converting enzyme inhibitors and angiotensin II receptor blockers) in pediatric patients with renal disease who are currently prescribed these classes of drugs.
- To determine the best housekeeping gene to be used in real time polymerase chain reaction (RT-PCR) by investigating the effect of inflammation on the expression profile of three housekeeping genes, glyceraldehyde-3-phosphate-dehydrogenase (GAPDH), β-actin and 18s ribosomal RNA (18s rRNA) in a rat model of pre-adjuvant arthritis.
- To determine the effect of inflammation on the expression profile of various drug targets and transporters in pre-adjuvant arthritis rat model.

Chapter 2

2. Effects of angiotensin II blockade on inflammation-induced alterations of pharmacokinetics and pharmacodynamics of calcium channel blockers*

2.1. Introduction

Inflammation is a normal response to external and internal stimuli. It is associated with release of inflammatory mediators such as pro-inflammatory cytokines and nitric oxide. These biochemical changes are associated with altered pharmacokinetics and/or pharmacodynamics of some cardiovascular drugs such as calcium channel[3, 4, 8] and β-adrenoceptor blockers[5, 6, 52]. For example verapamil potency is reduced in patients with active rheumatoid arthritis despite elevated drug plasma concentration[3]. Similar changes in verapamil action and disposition has been reported in aging[173] and obesity[1], two conditions, also, associated with elevated pro-inflammatory mediators[13, 14, 223]. Patients with arthritis, the elderly and obese are more prone to cardiovascular complications than the general population[224, 225]. In addition, inflammation is involved in the etiology of cardiac diseases such as acute myocardial infarction[11] and heart failure[12] and may contribute to poor therapeutic outcome. For example, elevated interleukin 6 (IL-6) in patients with unstable angina was linked to increased adverse coronary events[58].

* A version of this chapter has been published. Hanafy S, Dagenais NJ, Dryden WF and Jamali F, the British Journal of Pharmacology 2008; 135 (1): 90-99

The observed inflammation-induced reduction in response to verapamil, despite increased concentration, has been attributed to inflammation-induced reduction in binding to L-type calcium channels[4]. The reduced clearance of highly extracted drugs such as verapamil[3, 4, 8] and propranolol[6], observed in response to inflammation, is attributed to increased drug plasma protein binding[3, 4, 8] and/or reduced expression of the enzymes responsible for drug metabolism[8].

Inflammatory conditions do not reduce response to all cardiovascular drugs. The potency of angiotensin II receptor type I blockers (ARBs), valsartan[221] and losartan[222], is not reduced by rheumatoid arthritis. Indeed, for valsartan, a trend toward increased potency has been observed[221]. ARBs are known to have direct[226] and indirect[202] anti-inflammatory actions. The direct effect is likely through their free radical scavenging properties owing to their phenolic moiety[226] while the indirect action is through the inhibition of the pro-inflammatory effects of angiotensin II[202]. Owing to their anti-inflammatory properties, ARBs treatment may be a modality to alter the effect of inflammation on drug actions and disposition.

We used the pre-adjuvant arthritis (Pre-AA) model of inflammation[8]. This newly developed animal model allows studies under systemic inflammatory conditions in the absence of pain and stress associated with the fully developed adjuvant arthritis. Pre-AA is associated with elevated pro-inflammatory mediators concentration and depressed drug metabolic enzymes[8].

Similar to other inflammatory models, Pre-AA resulted in a significant reduction in the verapamil potency in prolonging PR interval (Figure 2-1)[9]. There was also a significant 74% reduction in the area under the effect curve (AUEC). The effect of valsartan treatment on verapamil pharmaocdynamics has been studied in our lab. Six days of treatment with valsartan (two daily doses of 30 mg/kg/dose) did not influence the response to a single oral dose of verapamil (25 mg/kg) in control rats. However, inflamed rats who were treated with valsartan responded to verapamil to a significantly greater extent than the untreated animals (Figure 2-2)[9]. Significantly greater verapamil potency and greater AUEC values (4-10 fold) were observed during 40-120 min post-dose in the inflamed-treated rats as compared with the inflamed-placebo (Figure 2-2)[9]. The aim of our work was to investigate whether this effect is also associated with normalization of the altered verapamil pharmacokinetics in pre-adjuvant arthritis rats and to study the meechanism behind valsartan interaction.

Our hypotheses were first, the diminished response to verapamil caused by inflammation is due to reduced calcium channel target protein. Second, the diminished potency of verapamil under inflammatory conditions is reversed by angiotensin II inhibition by reversing the downregulation of the target protein. Third, unlike pharmacodynamic changes, valsartan treatment does not reverse inflammation-induced changes in verapamil pharmacokinetics

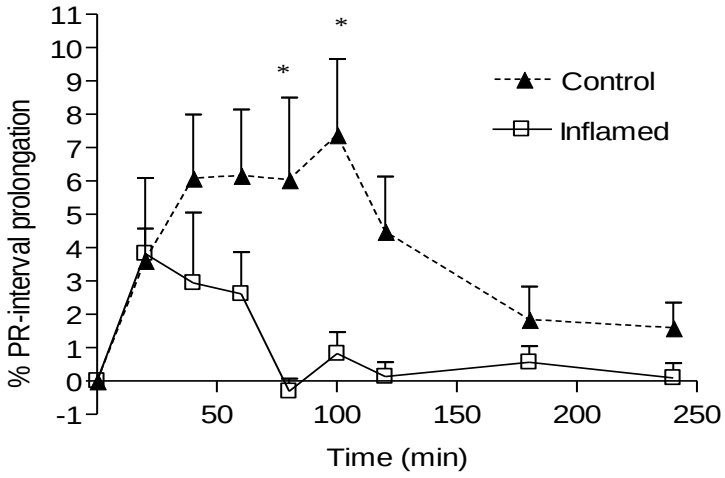


Figure 2-1. Verapamil-induced PR interval prolongation in Control and Inflamed rats (Pre-adjuvant arthritis) following a single oral 25 mg/kg dose of verapamil (n = 5/group). *, $p<0.05$ vs. Inflamed[9].

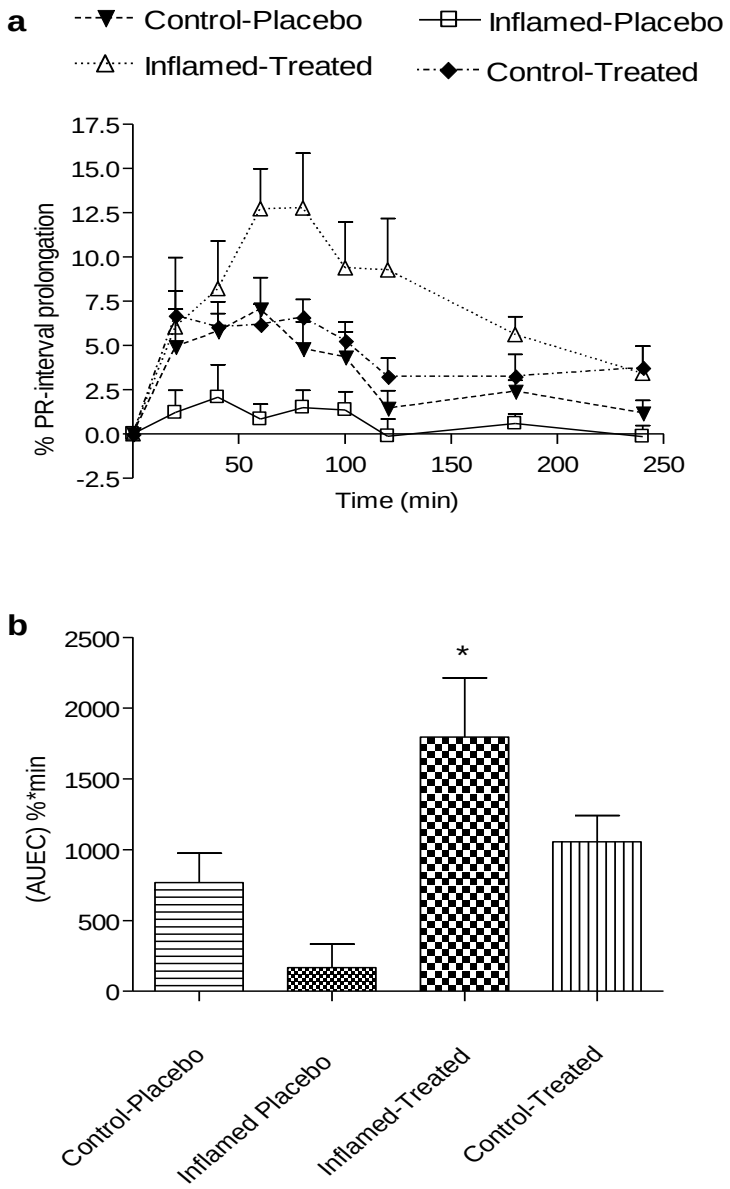


Figure 2-2. a, The effect of 6-days valsartan treatment on verapamil-induced PR interval prolongation in normal and inflamed rats following a single oral 25 mg/kg dose of verapamil (n = 8-9/group). Inflamed-Placebo vs Control- Placebo ($p < 0.05$ at 60 min); Inflamed-Placebo vs Inflamed-Treated ($p < 0.05$ at 40, 60, 80, 100, 120 min). b, The AUECs of the corresponding groups. *, $p<0.05$ vs. inflamed-placebo[9]

2.2. Methods

2.2.1. Materials

Racemic verapamil hydrochloride, the internal standard (+)-glaucine, heptafluorobutanol, Aspergillus nitrate reductase (10 U/ml), FAD, NADPH, lactic dehydrogenase (1500 U/ml), Tris base, Tris hydrochloride, protease inhibitor cocktail and pyruvic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). High-performance liquid chromatography (HPLC) grade hexane and HPLC grade isopropanol, 98 % ethanol, heptane and triethylamine were purchased from Caledon Laboratories (Georgetown, ON, Canada). Squalene was purchased from Kodak (Rochester, NY, USA). Polyethylene glycol 400 (PEG 400) was purchased from Wiler (London, ON, Canada). Heat-killed dried *Mycobacterium butyricum* was purchased from Difco (Detroit, MI, USA). Valsartan was supplied by Novartis Pharma (Basel, Switzerland). Losartan, a gift from Merck Research Laboratories (Rahway, NJ, USA), was used as the internal standard.

2.2.2. Experimental Animals

The study protocol was approved by the Health Sciences Animal Policy and Welfare Committee of the University of Alberta. A newly developed animal model of inflammation, 'Pre-adjuvant arthritis' (Pre-AA) was used in this study [8]. The experiments were carried out on male Sprague-Dawley rats (220-280 g). They were housed in a controlled-temperature room with a 12-hours dark/light cycles.

2.2.3. Pharmacokinetic Study

Animals (5-6/group) were randomized to 4 groups: Pre-AA treated with placebo (Inflamed-Placebo), Pre-AA treated with valsartan (Inflamed-Treated), control healthy rats treated with placebo (Control-Placebo) and control healthy rats treated with valsartan (Control-Treated). Pre-AA [8] was induced by injecting 0.2 ml of 50 mg/ml *Mycobacterium butyricum* suspended in squalene into the tail base of the inflamed groups. Control animals received an equal volume of normal saline into the tail base. The day of its injection was marked as day 0. From day 6 to 12, rats in the treated groups received valsartan. Oral 30 mg/kg valsartan suspended in PEG 400 were administered, via gastric gavage, every 12 hours. The placebo groups received a comparable volume of the vehicle only.

On day 11, the right jugular vein was cannulated in all animals for serial blood collections on day 12, while animals were under halothane/Oxygen anesthesia. Briefly a polyethylene cannula (Dow Corning Corp., Midland, MI, USA) tipped with 2 cm of Silastic tubing (Becton Dickinson, Sparks, MD, USA) was inserted into the right jugular vein and exteriorized by subcutaneous tunneling to an incision made in the interscapular area. On day 12, two hours following valsartan dose, a single oral dose of 25 mg/kg verapamil was administered orally to all groups. Plasma 0.5 ml samples were collected at 0, 20, 40, 60, 120 and 240 min post dosing for plasma verapamil and nor-verapamil

analysis. Valsartan plasma concentrations were measured 2 h post-dose only in the valsartan treated control and inflamed groups.

2.2.4. Stereospecific Verapamil and Nor-verapamil Assay

A previously reported assay[227] was used. A 100 µl of plasma sample or standard was added to 75 µl of the internal standard (+)-glaucine (400 ng/ml), followed by 100 µl of 2 M sodium hydroxide and 0.4 ml of phosphate buffer (pH 7, ionic strength 0.1). Aliquots of 6 ml of heptane/heptafluorobutanol (99:1) were added and vortex mixed for 1 min. The samples were centrifuged at 2000 g for 10 min. The organic layers were transferred to clean tubes and evaporated to dryness. Residuals were reconstituted in 200 µl of mobile phase (hexane: ethanol: isopropanol: triethylamine, 92:4:4:0.1 v/v) and injected (100 µl) into the HPLC at a flow rate of 0.7 ml min^{-1}. Peaks were resolved using a Chiralpak AD-H column (Daicel Chemical Inc., Tokyo, Japan) a chiral column and detected at excitation and emission wavelengths of 272 and 317 nm, respectively. Standard curves were linear over the range of 10 to 2000 ng/ml (CV < 10 %). Since standard nor-verapamil was not available, plasma nor-verapamil is presented as peak area ratio. The area under the plasma drug or metabolite concentration curve (AUC) was calculated using the log-linear trapezoidal method.

2.2.5. Valsartan Assay

A previously reported assay was used to analyze plasma valsartan concentration [228]. A 100 µl of plasma sample or standard was added to 100 µl of the internal

standard losartan (5 µg/ml), followed by acidification with 125 µl of 1 M phosphoric acid. Aliquots of 10 ml of methyl-*tert*.-butyl ether were added and vortex mixed for 3 min. The samples were centrifuged at 2000 g for 5 min. The organic layers were transferred to clean tubes containing 200 µl of 0.05M sodium hydroxide and again vortex mixed for 2 min and centrifuged at 2000 g for 5 min. The organic layer was discarded and the aqueous layer is acidified with 75 µl 0.2M phosphoric acid. Aliquots of 125 µl of the aqueous layer were injected into the HPLC at a flow rate of 1.3 ml min^{-1}. The mobile phase was 70 % phosphate buffer pH 2.8 and 30 % acetonitrile. Peaks were resolved using a C_{18} analytical column (Phenomenex, Torrance, Mississauga, Canada) attached to a NovaPak C8 Guard-Pak HPLC Pre-column insert (Waters, Millipore, Mississauga, Canada) and detected at excitation and emission wavelengths of 265 and 378 nm, respectively. Standard curves were linear over the range of 0.5 to 5 µg/ml (CV < 12 %).

2.2.6. Serum Nitrite Analysis

Nitric oxide (NO) concentration was indirectly measured through concentrations of serum nitrite and nitrate, its stable breakdown products, using a previously reported method [229]. Nitrate was reduced into nitrite by incubating 100 µl sample or standard with 10 µl of Aspergillus nitrate reductase (10 U/ml), 25 µl of 0.1 mM FAD, 50 µl 1mM NADPH, 25 µl of 1M HEPES (pH 7.4) and 290 µl of deionized water for 30 min at 37^{o} C. This was followed by adding 5 µl of lactate dehydrogenase (1500 U ml^{-1}) and 50 µl of 100 mM pyruvic acid and re-incubation

for another 10 min at 37^{o} C. Subsequently, 1 ml of Griess reagent (0.2 % naphtylene ethylene diamine and 2 % sulphanilamide in 5 % phosphoric acid) was added and incubated for 10 min at room temperature. The absorbance of the developed color was measured at 543 nm using a Vmax Molecular Devices plate reader (Bio-Tek Instruments Inc., Winooski, VT, USA). Standard curves were linear over the range of 6.25 to 200 μM (CV < 20 %).

2.2.7. Heart Membrane Preparations

Heart membrane preparations were obtained using a previously used method [4] after minor modifications. Another four groups of rats (4/group) similar to those described under "Pharmacokinetic Study" were used without animal cannulation. Instead, on day 12, rats were anesthetized with halothane/Oxygen, the thoracic cavity of the rats was opened, blood was removed by cardiac puncture and the heart was excised and placed in ice-cold Tris-Buffer (0.05 M, pH 7.4). Hearts were then weighed and cut into small pieces and put into an ice-cold Tris buffer/protease inhibitor cocktail (19:1) mixture (10 ml/g wet tissue), followed by homogenization in a tube immersed in ice using Brinkmann Homogenizer (Kinematica AG, Switzerland) for 30 sec. The crude homogenate was then centrifuged at 5000 g at 4^{o} C for 10 min to disrupt nuclei and cytoskeleton particles. Aliquots of 100 μl were taken from the supernatant (S9 fraction) for Western blotting and stored at -80^{o} C. The remainder was centrifuged at 100,000 g at 4^{o} C for 1 hr. The resultant pellet was re-suspended in 10 ml Tris buffer (pH 7.4) and aliquots were stored at -80^{o} C for the radioligand binding study.

The amount of protein in samples was determined using the method of Lowry [230] using a commercially available protein assay kit (Biorad Laboratories, Hercules, CA). A 250 µl of 10 % Folin reagent were added to a mixture of 5 µl sample and 25 µl of alkaline copper tartarate solution. The developed color was analyzed using spectrophotometry at 570 nm using a Vmax Molecular Devices plate reader (Bio-Tek Instruments Inc., Winooski, VT). Standard curves were linear over concentration range between 0.2-1.44 mg ml^{-1} (CV<10 %).

2.2.8. Equilibrium Radioligand Binding Study

A previously reported method[4] was used following minor modifications. ^{3}H-nitrendipine was used at serial dilutions of 0, 0.025, 0.05, 0.1, 0.2, 0.3, 0.4 and 0.8 nM in a final volume of 500 µl in Tris buffer (pH 7.4, 0.05 M). The reaction was initiated by adding 100 µg protein of the rat heart membrane preparation in the presence and absence of an excess cold ligand ($4x10^{-5}$ M nifedipine) for non-specific and total binding, respectively and then incubated for 90 min. The reaction was terminated by rapid filtration through Whatman Gf/B filters that were soaked previously with Tris buffer. Filters were then immersed in plastic tubes containing 4 ml CytoScint liquid scintillation cocktail (ICN Biochemicals, Costa Mesa, CA, USA) followed by shaking for 30 sec. Beckman LS 6500 multi-purpose liquid scintillation counter (Fullerton, CA, USA) was used to measure the radioactivity retained in each filter. The specific binding was determined by subtraction of the non-specific binding from the total binding. The data analysis,

non-linear curve fitting, determination of the equilibrium dissociation constant (*K*) and the maximum number of binding sites (B_{max}) were carried out using Prism software v. 4.03 (GraphPad Software, Inc., San Diego, CA, USA).

2.2.9. Western Blot Analysis

Western immunoblot was used to determine the protein concentration of the $Ca_v1.2$ subunit of L-type calcium channels in the heart. The cardiac proteins were denatured by boiling at 100^oC for 5 min followed by SDS-gel electrophoretic separation of 50 µg aliquots on 7.5 % acrylamide for 1 h at 200 volts. The resultant separation was transferred to a nitrocellulose membrane. The membrane was incubated overnight in a blocking solution (2 % bovine serum albumin, 5 % skim milk and 0.05 % Tween 20 in Tris-buffered saline) to block the nonspecific binding. Nitrocellulose membranes were incubated with the primary antibody (1:400 dilution of polyclonal rabbit anti-calcium channel ($Ca_v1.2$ subunit) (Sigma-Aldrich, St. Louis, MO, USA) or 1:1000 dilution of polyclonal rabbit anti-β actin (Abcam Inc., Cambridge, MA, USA) with shaking for 2 h followed by the secondary antibody (1:7500 dilution of horseradish peroxidase (HRP)-conjugated goat antirabbit IgG antibody) (Biorad Laboratories, Hercules, CA, USA) for 1 hr. The primary antibody is raised in rabbits against the amino acids 848 to 865 of rat Cav1.2. The resultant interaction was detected by chemiluminescence (ECL Western Blotting Detection reagents, Biorad Laboratories, Hercules, CA, USA) and the bands density was measured using Image J software (National Institute of Health, Bethesda, MD, USA).

2.2.10. Data Analysis

Data are expressed as mean ± standard error of the mean (SEM). Differences among the four study groups were assessed using one-way (ANOVA) followed by Bonferroni post-test. To assess the homogeneity of variance and normality of the data, Bartlett's test and Kolmogorov-Smirnov test were used, respectively. A *p* value of less than 0.05 was considered statistically significant. Satatistics were analyzed using Prism software v. 4.03 (GraphPad Software, Inc., San Diego, CA, USA).

2.3. Results

2.3.1. Verapamil and Nor-verapamil Pharmacokinetics

Table 2-1 depicts pharmacokinetic indices for both verapamil and nor-verapamil enantiomers in all groups. Plasma verapamil enantiomer concentrations were significantly elevated in inflamed groups as compared to the control groups. Both, the area under the curve ($AUC_{0\text{-}4h}$) and the maximum plasma concentration (C_{max}) for the enantiomers in inflamed animals were significantly higher by many magnitudes (Figures 2-3 a and b). Valsartan treatment had no significant effect on the pharmacokinetics of S-verapamil in the presence or absence of inflammation. However, it significantly raised the area under the curve of R-verapamil in inflamed animals.

Valsartan treatment was associated with a significant rise in the plasma concentration of both nor-verapamil enantiomers in inflamed animals. The plasma concentrations in treated animals were approximately 3 times higher than those in both inflamed-placebo and control groups (Figures 2-3 c and d).

2.3.2. Valsartan Plasma Concentration

As depicted in Table 2-1, valsartan concentration, observed 2 h post-dosing, did not significantly differ between the control and the inflamed groups.

2.3.3. Serum Nitrite Concentration

Serum nitrite concentration measured on Day 12 which was significantly elevated by inflammation was not normalized by valsartan (Table 2-1).

2.3.4. ^{3}H-Nitrendipine Binding to Cardiac L-Type Calcium Channels

Results are depicted in Figure 2-4. Inflammation caused a significant reduction in the binding capacity of ^{3}H-nitrendipine to L-type calcium channels in rat heart (Bmax in inflamed-placebo and control-placebo groups were 3.1±0.7 and 10.8±2.0 fmol/mg protein, respectively). On the other hand, there were no significant changes in the equilibrium dissociation constant *K* (Control placebo 0.25±0.07 vs. inflamed placebo 0.18±0.06 nM) or Hill coefficient n_H (Control placebo 2.0±0.8 vs. inflamed placebo 1.7±0.8). Valsartan treatment caused a significant increase of ^{3}H-nitrendipine binding as shown by returning Bmax back to normal (10.8±2.5 fmol/mg protein) without affecting *K* (0.35±0.1 nM) and N

(2±0.7). Normal rats treated with valsartan did not show any further increase in Bmax (9.5±2.3 fmol/mg protein).

2.3.5. Western Blot of $Ca_v1.2$ Subunit of Cardiac L-type Calcium Channels

Density of the low molecular weight $Ca_v1.2$ subunit (190 KD) was significantly reduced in inflamed animals by 48% as compared with control rats (Figure 2-5). The mean high molecular weight subunit (210 KD) was reduced by (41%) but the reduction was not significant. Valsartan treatment caused a significant correction of the density of the low molecular weight $Ca_v1.2$ subunit back to normal. Moreover, there was a significant direct correlation between the low molecular weight subunit density and the observed Bmax (Figure 2-6).

Table 2-1. Effect of inflammation and valsartan on serum nitrite and pharmacokinetic indices following a single oral dose of 25 mg/kg racemic verapamil and six days of treatment with 30 mg/kg oral dose of valsartan or placebo (n= 5-6/group).

Group	Control-Placebo	Inflamed-Placebo	Inflamed-Treated	Control-Treated
Cmax (µg/ml)				
S-verapamil	0.45 ± 0.1^{a}	5.8 ± 2.7^{b}	5.7 ± 0.9^{b}	0.74 ± 0.5^{a}
R-verapamil	0.2 ± 0.1^{a}	1.2 ± 0.3^{b}	2.0 ± 0.5^{b}	0.13 ± 0.1^{a}
AUC0-4 h (µg min/ml)				
S-verapamil	65 ± 14^{a}	$616 \pm 183^{b,c}$	1039 ± 266^{b}	$270 \pm 212^{a,c}$
R-verapamil	21 ± 7^{a}	124 ± 19^{b}	283 ± 73^{c}	$33 \pm 21^{a,b}$
AUC0-4 h (area ratio min)				
S-nor-verapamil	149 ± 12^{a}	431 ± 80^{a}	1029 ± 289^{b}	243 ± 157^{a}
R-nor-verapamil	20 ± 4.5^{a}	190 ± 34^{a}	633 ± 171^{b}	67 ± 43^{a}
Mean valsartan plasma concentration 2h post-dosing (µg/ml)	ND	ND	2.9 ± 1.3^{a}	1.5 ± 0.26^{a}
Nitrite (µM)	31.6 ± 2.9^{a}	138 ± 17^{b}	145 ± 22^{b}	29.4 ± 2.1^{a}

Values are shown as mean (±SEM). Different superscript letters (a, b and c) on the values of each variable (each row) denote significant difference ($p<0.05$) while similar letters are not significantly different. ND, not determined.

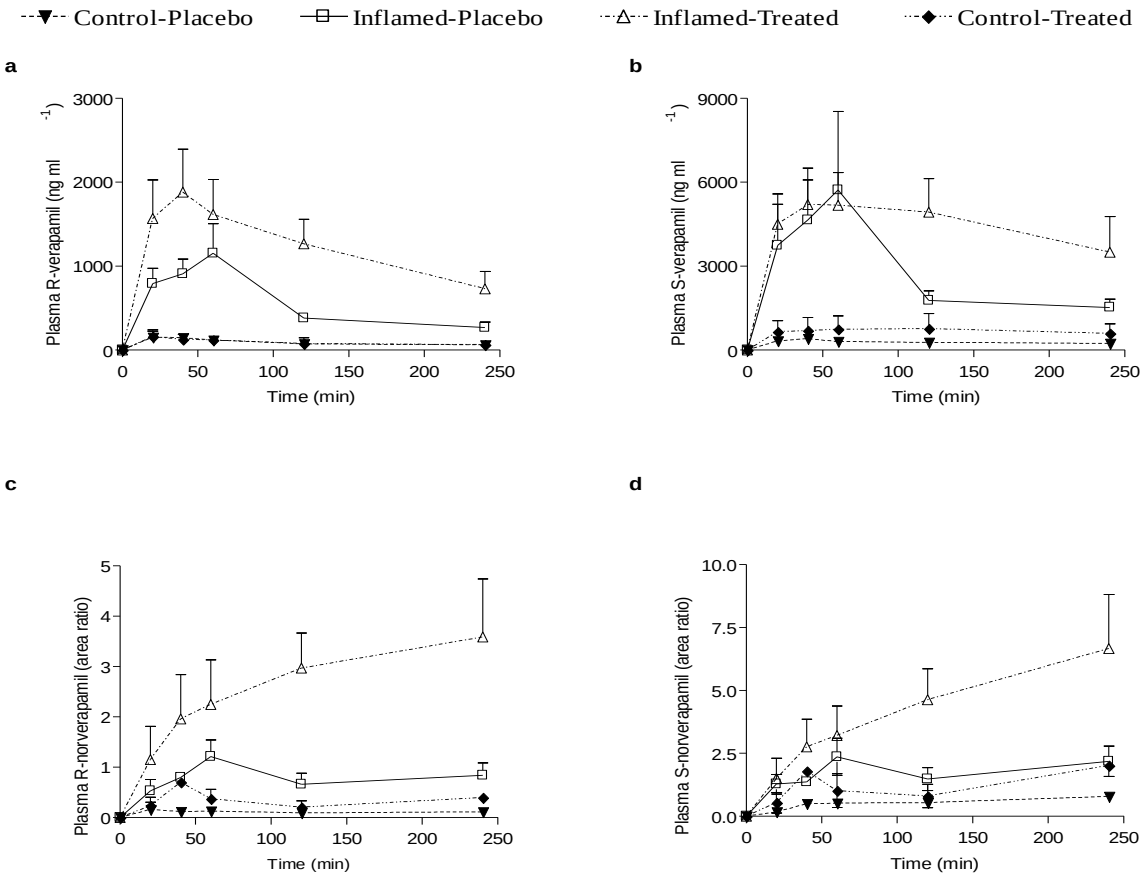


Figure 2-3. Effect of 6-days valsartan treatment on plasma concentration-time profile of both verapamil and nor-verapamil enantiomers in normal and inflamed rats following a single oral 25 mg/kg dose of verapamil (n = 5-6/group).

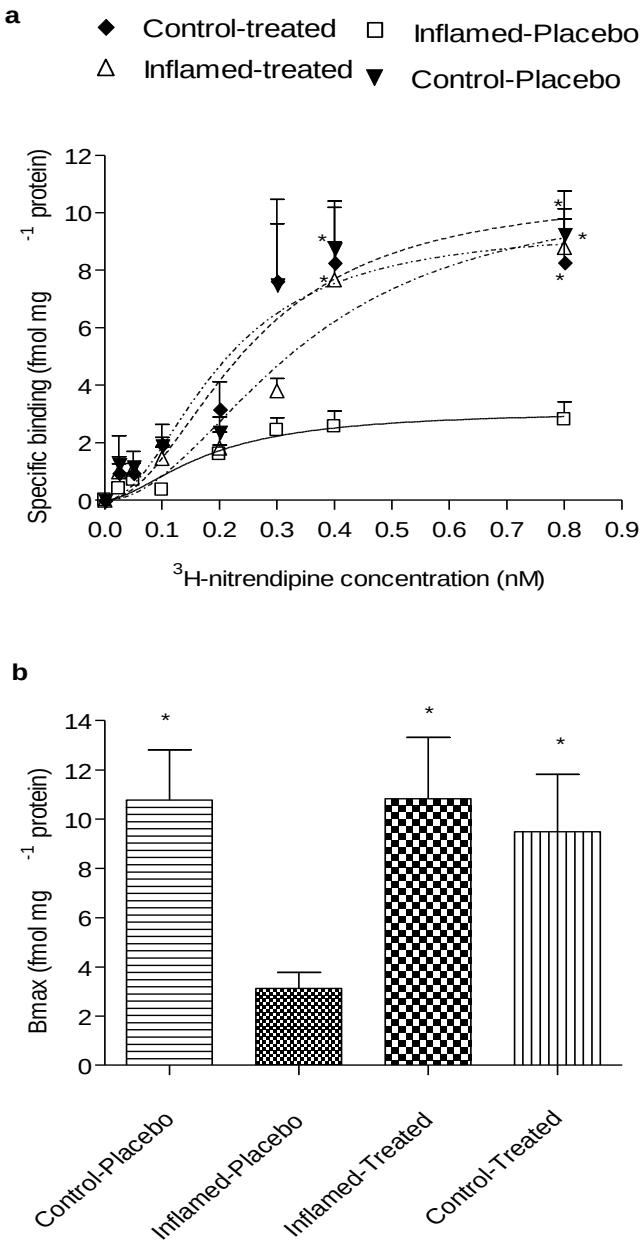


Figure 2-4. Effect of 6-days valsartan treatment on ^{3}H-Nitrendipine binding to L-type calcium channels in rat cardiac cell membrane preparations (n=4/group). a, Binding obtained at increasing ligand concentrations. b, Mean Bmax in the four groups. * $p<0.001$ vs. inflamed-placebo.

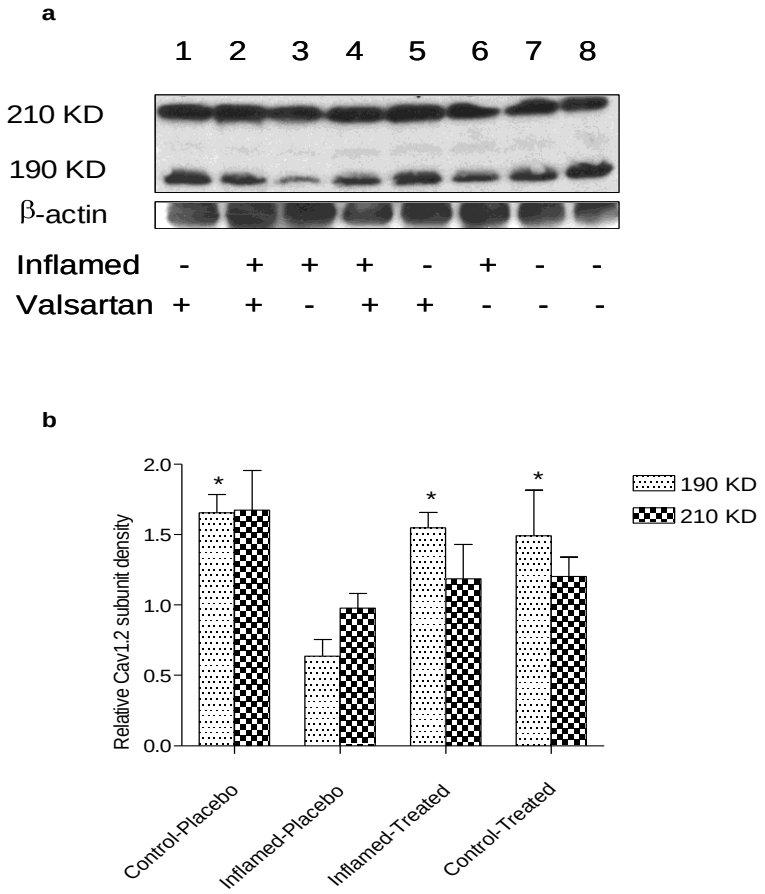


Figure 2-5. The effect of 6-days valsa rtan treatment on cardiac $Ca_v1.2$ subunit density in rat hearts (n=4/group). a, Western blot of the $Ca_v1.2$ subunit in rat heart. b, Density of the low (190 KD) and high (210 KD) molecular weight forms (190 KD) in the four groups. The bands density was measured using Image J software (National Institute of Health, Bethesda, MD, USA). * $p<0.05$ vs. inflamed-placebo.

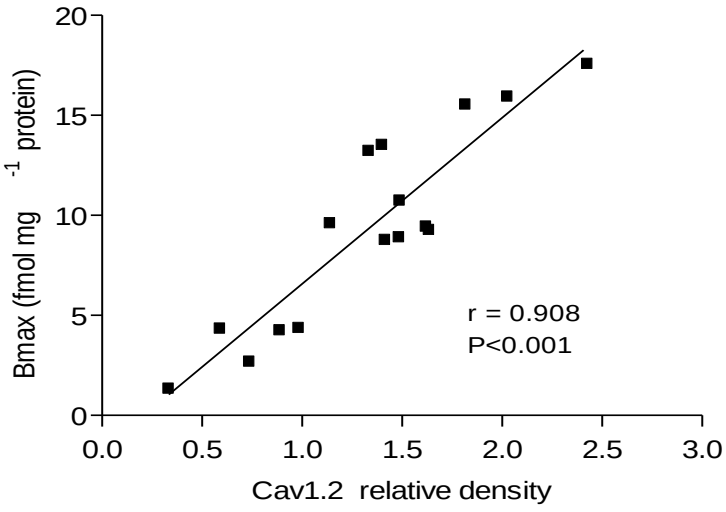


Figure 2-6. Correlation between the low molecular weight $Ca_v1.2$ subunit density and the observed maximum binding (Bmax). (n=16)

2.4. Discussion

Our present data confirm previous reports that inflammatory conditions alter the pharmacokinetics and pharmacodynamics of verapamil in both humans[1, 3, 173] and rats[3, 4, 8, 91]. Downregulation of CYP450 and/or increased protein binding are believed to be responsible for the increased concentration of the drug[8]. Despite its elevated plasma concentration, verapamil-induced PR interval prolongation is significantly reduced in inflamed rats compared to control[9]. This has been explained by reduced binding to L-type calcium channels[4]. Here for the first time, we present data suggestive of reduced target protein density (Figure 2-5) as an explanation for the reduced binding [4], hence reduced potency [3, 4].

The effect of inflammation on the pharmacokinetics and pharmacodynamics is associated with significant rises in pro-inflammatory mediators[4, 5, 8]. Verapamil clearance and response are both reduced in human arthritis[3], aging[173] and obesity[1]; three conditions associated with elevated concentrations of pro-inflammatory mediators. Angiotensin II has been implicated as a powerful pro-inflammatory mediator [202]. It stimulates the production of reactive oxygen species as hydrogen peroxide and superoxides[210]. Consequently, the resultant oxidative stress activates the intracellular transcription protein nuclear factor kappa B (NFκB)[148]. The latter, in turn, is translocated to the nucleus to act on specific response elements on DNA modulating the expression of some proteins as pro-inflammatory mediators[149]. L-type calcium channels appear to be

regulated by this mechanism as TNFα-induced NFκB activation causes its downregulation[150]. Accordingly, the treatment with valsartan, an angiotensin II receptor antagonist reversed the downregulating effect of inflammation on verapamil response[9]. This coincided with normalization of the binding capacity (Figure 2-4) and density (Figure 2-5) of the low molecular weight $Ca_v1.2$ subunit of the target protein. We do not know the exact mechanism of reversal of the effect of inflammation by valsartan. It is known, however, that angiotensin II receptor antagonists have anti-inflammatory properties[202]. They have been shown to be of benefit in atherosclerosis, heart failure, and diabetes. Much of this benefit is independent of the anti-hypertensive effect of angiotensin II interruption suggesting a unique protective mechanism.

We have previously reported that NO is a reliable marker of inflammation in both humans[3] and rats[4], hence, we used nitrite, an stable metabolite of NO, as one of the measures of inflammation. It has been reported that the serum nitrite is significantly elevated in inflammation in parallel with serum tumor necrosis factor concentrations[8]. Interestingly, the valsartan treatment of inflamed rats did not normalize the observed elevated nitrite concentration; i.e., the improved verapamil pharmacodynamics in treated animals was independent of serum NO. This may suggest that the angiotensin II blockade may insert its normalizing effect through increased expression of the anti-inflammatory mediators rather than inhibition of the pro-inflammatory mediators.

The observed altered binding of the calcium channel blocker ^{3}H-nitrendipine to the target protein was only associated with altered Bmax which was normalized by valsartan as the binding affinity (*K*) was not significantly altered. Thus, the changes in the binding may be explained by a non-competitive inhibition and/or a change in the target protein density. The former can be ruled out since both reduced binding caused by inflammation and reversal of the downregulating effect by valsartan were parallel with changes in the density of the low molecular weight $Ca_v1.2$ subunit protein (Figure 2-6).

Equilibrium radioligand binding studies were performed using ^{3}H-nitrendipine because it is a specific L-type calcium channel ligand and its electrophysiological effects have been found to be parallel to verapamil [231, 232].

The Western blot presentation of the functional $Ca_v1.2$ subunit of cardiac L-type calcium channels has been reported to exhibit two bands representing a low (190KD) and high (210KD) molecular weight molecules [233]. It has been believed that each band is associated with a different physiological function [139, 233, 234]. Our data revealed a positive and significant correlation between the calculated Bmax values and the low but not the high molecular weight band (Figure 2-6). The parallel changes in the binding to the target protein (Figure 2-4) and the density of the 190 KD band (Figure 2-5 and 2-6) observed in our study, suggest that the low molecular weight subunit may be more relevant in the

context of the present work' i.e., the mechanism of involvement of inflammation and valsartan in altering pharmacological response to verapamil.

Six days treatment with valsartan was sufficient to elevate the reduced channel density. Indeed, there is evidence that L-type calcium channel expression takes place very rapidly even within 2 hours[235]. Moreover, other in vitro studies have observed the reduced expression of the functional α subunit in hyperthyroidism[142], heart failure[134] and atrial fibrillation[143, 144] indicating that it is a highly regulated protein.

The downregulating effect of inflammation does not only reduce density of L-type target proteins (Figure 2-5) as it also reduces the density of the enzyme responsible for clearance of verapamil[8]. Despite reversal of the pharmacodynamic effect of verapamil, valsartan did not return the elevated verapamil plasma concentration back to the range noticed in normal rats (Figure 2-3). This may indicate a different mechanism for the reduced enzyme density as compared to either that for the L-type calcium channel target protein, and/or the need for a longer duration and larger doses of valsartan. Another plausible explanation is that valsartan may have its own inhibitory effect on the clearance of verapamil which may hamper the return to normal caused by the anti-inflammatory effect of the drug. Indeed, in the inflamed rats, the treatment with valsartan resulted in elevation of plasma verapamil enantiomer concentrations which was only significant for the R enantiomer (Table 2-1, Figure 2-3a). Nor-verapamil

enantiomers concentrations were also significantly increased in inflamed rats (Table 1, Figure 2-3 b and c). Interestingly, however, no verapamil-valsartan pharmacokinetic interaction was noticed in control rats. This may be explained by the abundance of the enzyme in the healthy animals as compared to the inflamed rats. Valsartan binds to CYP2C enzyme family that O-demethylate verapamil and nor-verapamil[170, 172] but only with a moderate affinity[236, 237], In healthy rats, therefore, the inhibitory effect of valsartan on the CYP2C enzymes may be negligible. This effect may become pronounced in inflamed animals as the drug metabolizing enzymes are relatively scarce.

Although R-verapamil and nor-verapamil enantiomers have some calcium channel blocking effect, the main pharmacological relevance of the drug is attributed to the S enantiomer[175]. The contribution of the pharmacokinetic interaction between valsartan and R-verapamil and nor-verapamil to the observed enhanced verapamil potency in valsartan treated-inflamed rats is, therefore, unlikely to be significant.

Valsartan plasma concentration 2h post-dosing was not altered by inflammation which is in close agreement with that have been observed in humans[221]. Hence, the observed effects of valsartan are not due to changes in its plasma concentration.

The present data demonstrate, for the first time, the effects of the angiotensin II receptor type I antagonism on the pharmacodynamic profile of calcium channel blockers under an inflammatory condition. This beneficial interaction of valsartan with calcium channels blockers, once also proven in humans, may be of value in cardiac patients with superimposing inflammatory diseases such as rheumatoid arthritis.

Moreover, angiotensin II antagonists may increase the L-type calcium channel expression in conditions that are associated with reduced calcium channel expression.

Chapter 3

3. Drug-disease interaction: Reduced verapamil response in isoproterenol-induced myocardial injury in rats[†]

3.1. Introduction

Inflammation is involved in the pathogenesis and progression of cardiovascular diseases such as hypertension [78], heart failure [12] and acute myocardial infarction (AMI) [11]. Increased inflammatory mediator concentration in acute coronary syndrome patients is associated with poor disease prognosis, prolonged hospital stay and high mortality rate [57, 58]. This may, at least, in part, be explained by reduced drug response and treatment failure since animal and human studies have shown reduced potency of some cardiovascular drugs such as calcium channel [3, 4, 8] and β-adrenoceptor blockers [5, 6, 52] in the presence of systemic inflammation. For example, calcium channel blockers potency is reduced in patients with active rheumatoid arthritis [3], aging [173] and obesity [1, 238]. These conditions are associated with elevated pro-inflammatory mediators [14, 15, 187]. The reduced response has been attributed to inflammation-induced reduction in binding to L-type calcium channels [4, 9] and downregulation of calcium channel target protein [9]. With myocardial injury as in myocardial infarction, in addition to the associated inflammatory condition, the expected loss of viable tissues and reduced cellular

[†] A version of this chapter has been published. Hanafy S, El-Kadi AO and Jamali F. Pharmacology 2010; 86: 196-202

proteins [239] may also be associated with a parallel loss of drug-target proteins, thereby, reduced response to verapamil.

The purpose of this study was to investigate whether pharmacodynamics of verapamil are altered as a consequence of acute myocardial injury in parallel to what has previously been observed in other type of inflammatory conditions [1, 3, 4, 9, 173]. Our hypotheses were 1) verapamil potency is reduced in rats following myocardial injury, and 2) the decreased verapamil potency is due to inflammation-induced downregulation of the cardiac target protein. We used isoproterenol (ISP)-induced myocardial injury rat model [240-244], a model in which infarct-like lesions in the myocardium are produced demonstrating biochemical, electrocardiographic and morphologic features similar to those observed in acute myocardial infarction [240-244].

3.2. Methods

3.2.1. Experimental Animals

The study protocol was approved by the Health Sciences Animal Policy and Welfare Committee of the University of Alberta. Animals were treated according to the Canadian Council on Animal Care Guidance. Experiments were carried out on male Sprague-Dawley rats (230-280 g). They were housed in a controlled-temperature room with a 12-hours dark/light cycles.

3.2.2. Experimental Protocol

Two groups of rats were divided into control (n= 8) and post isoproterenol-induced myocardial injury referred to as (post-AMI) (n= 13). Myocardial injury was induced by two 24-h apart subcutaneous doses of 150 mg/kg isoproterenol HCl solution (120 mg/ml normal saline) (days 1 and 2). Control rats received a comparable volume of normal saline. Subsequently, modified ECG leads were implanted subcutaneously over the xyphoid process and in the right and left axilla while the animals were under halothane/oxygen anesthesia. Development of cardiac necrosis was confirmed by the presence of pathologic Q-waves (Q-waves with amplitude equal to or higher than 25% of R-wave), ST-segment elevation above the baseline and/or reduction of R-wave amplitude by more than 25%. In addition, as a biomarker of myocardial damage (released as a result of cellular breakdown), serum cardiac troponin I (cTnI) was also determined. On day 4, each rat was dosed with 25 mg/kg p.o. of verapamil HCl solution and ECG measurements were made at 0, 20, 40, 60, 80, 100, 120, 180 and 240 min post-dosing. PR interval and heart rate (HR) were measured. The ECG device was MP100 system (Biopac Systems Inc., Goleta, CA, USA). Data were recorded using Acknowledge Software (World Precision Instruments, Miami, FL, USA). Verapamil response was calculated as the area under the effect curve (AUEC) using the linear trapezoidal method by taking the percent change of PR interval and HR from baseline and multiplying by the time interval in min (% min).

Maximum effect (Emax) percentage was also recorded. Subsequently, animals were euthanized, hearts excised and blood samples collected for analysis of serum tumor necrosis factor α (TNF-α), interleukin 4 (IL-4), interferon-γ (IFN-γ) and nitrite (NO2). Verapamil enantiomers plasma concentrations in sample taken 4.5 h post-dose were measured using a stereospecific a previously reported method[227] described in Chapter 2.

3.2.3. Mediators Determination

Serum cTnI was determined using a commercially available ELISA kit (Life Diadnostics Inc., West Chester, PA, USA). Serum TNF-α, IFN-γ and IL-4 were also determined using ELISA kits (Abcam Inc., Cambridge. MA, USA), according to the manufacturer's instructions. Nitric oxide (NO) was indirectly measured by analyzing serum nitrite, its stable breakdown product, using Grisham's method[229] described in Chapter 2.

3.2.4. Western Blot Analysis

$Ca_v1.2$ subunit protein expression in the rat heart was determined using Western blot analysis. Heart tissue samples were prepared using a previously reported method [4] after minor modifications. The excised hearts described under "Section 3.2.2. Experimental Protocol" were weighed and cut into small pieces. This was followed by adding an ice-cold Tris buffer (pH 7.4): protease inhibitor cocktail (19:1) mixture (10 ml/g wet tissue) to each heart and homogenizing the mixture in a tube immersed in ice using Brinkmann Homogenizer (Kinematica AG,

Switzerland) for 30 sec. The crude homogenate was then centrifuged at 750 g at 4^{o}C for 5 min to disrupt nuclei and cytoskeleton particles. The supernatant was then centrifuged at 10000 g at 4^{o} C for 20 min. The resultant supernatant was then stored at -80^{o} C until used to measure the target protein. Samples protein concentrations were determined using Lowry's method [230] using a commercially available protein assay kit (Biorad Laboratories, Hercules, CA, USA).

Cardiac proteins were denatured by boiling heart samples at 100^{o} C for 5 min followed by SDS-gel electrophoretic separation of 50 μg aliquots on gradient polyacrylamide gel (4-20%) (Biorad Laboratories, Hercules, CA, USA) for 90 min at 200 volts. The resultant separation was transferred to a nitrocellulose membrane. The membrane was incubated overnight in a blocking solution (2% bovine serum albumin, 5% skim milk and 0.05% Tween 20 in Tris-buffered saline) to block the nonspecific binding. Nitrocellulose membranes were incubated with the primary antibody (1:200 dilution of polyclonal rabbit anti-$Ca_v1.2$ (Chemicon International Inc., Temecula, CA, USA) or 1:1000 dilution of polyclonal rabbit anti-β actin (Abcam Inc., Cambridge, MA) with shaking for 2 h followed by the secondary antibody (1:7500 dilution of horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG antibody) (Biorad Laboratories, Hercules, CA) for 1 hr. The resultant interaction was detected by chemiluminescence (ECL Western Blotting Detection reagents, Biorad Laboratories, Hercules, CA, USA) and the bands density was measured using Image J software (National Institute of Health, Bethesda, MD, USA).

3.2.5. Real time PCR

To determine the potential changes happening at the transcriptional level of the gene coding for $Ca_v1.2$, real time polymerase chain reaction (PCR) was carried out using three groups of rats (four per group): Control (C), ISP-Day 2 (D2) and ISP-Day 4 (D4). AMI rats were injected with two daily doses of isoproterenol as described under "Experimental Protocol". Groups C, D2 and D4 rats were then euthanized and their hearts were excised and instantly frozen in liquid nitrogen, on days 1, 2 and 4, respectively. The excised hearts were stored in -80 degrees.

Total RNA was isolated from the frozen hearts using TRIzol® reagent (Invitrogen, Carlsband, CA, USA) according to the manufacturer's instructions. A 0.25 g of the frozen tissue was homogenized on ice in a tube containing 3 ml of TRIzol® reagent. Then, 800 µl aliquots of the homogenate were transferred to eppendorf tubes containing 160 µl chloroform followed by shaking by gentle inversion for 30-45 sec. After leaving the samples to separate for 3-5 min, they were centrifuged for 15 min at 12000 rpm at 3-4 °C. The supernatants were then transferred to eppendorf tubes where 400 µl of isopropyl alcohol was added. The tubes were then stored at -20 °C overnight. In the following day, after centrifuging the samples at 12000 rpm for 10 min at 4 °C, the supernatants were discarded and the resultant white pellets were washed with 500 µl of 75% ethanol in DEPC (diethylpyrocarboncate) water. Finally, the pellets were reconstituted with an appropriate volume of DEPC water to provide a suitable working RNA

concentration. The samples were stored in -80 °C. This was followed by spectrophotometric quantitation of the isolated RNA by measuring the absorbance at 260 nm.

cDNA was synthesized from 1.5 µg total RNA samples with the random primers scheme using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). The reverse transcription master mix was prepared on ice. The master mix per reaction included 2 µl of 10X reverse transcription buffer, 0.8 µl of 25X dNTP Mix (a nucleotides mixture), 2 µl of 10X random primers, 1 µl of Multiscribe^{TM} reverse transcriptase and 4.2 µl of autoclaved water. A 10 µl of the master mix was mixed with an equal volume of the sample containing 1.5 µg total RNA and then the mix was placed in the thermal cycler. The thermal cycler conditions were 25 °C for 10 min at, 37 °C for 120 min, 85 °C for 5 sec and then 4 °C.

Real Time PCR was performed on an ABI 7500 Real Time PCR system (Applied Biosystems, Foster City, CA, USA). RT-PCR mixture per reaction included 12.5 µl of SYBR Green PCR Master Mix, 0.075 µl of forward primer, 0.075 µl of reverse primer, 11.15 µl of autoclaved water and 1.25 µl of the cDNA sample. PCR cycles conditions were 95°C for 15 sec (denaturation) followed by 60 sec at 60 °C (annealing and extension). The PCR cycle was repeated 40 times. The primers for $Ca_v1.2$ were forward 5'- AGC AAC TTC CCT CAG ACG TTT G -3' and reverse 5'- GCT TCT CAT GGG ACG GTG AT -3' [245, 246]. The

housekeeping gene tested was the rat GAPDH: forward primer 5'- CAA GGT CAT CCA TGA CAA CTT TG -3' and reverse 5'- GGG CCA TCC ACA GTC TTC TG -3'. Folds of mRNA changes normalized to GAPDH were determined. Melting curves were carried out to confirm amplification of single sequences and absence of primer dimers. Primers were purchased from Integrated DNA technologies (Coralville, IA, USA). PCR products were produced and detected quantitatively using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA). PCR data were analyzed using delta delta C_T method ($\Delta\Delta C_T$) as described by Livak and Schmittgen[247]. The data are expressed as fold change relative to a calibrator (Control) and normalized to the housekeeping gene.

3.2.6. Data Analysis

All data are expressed as mean (n=8/group) ± SEM, except for verapamil concentrations that are presented as means (n=8/group) ± SD. Statistical significance between the control and post-AMI groups was analyzed using a two-tailed Student's t-test. The time-course effect data were analyzed using the two-way ANOVA. ECG changes from day 1 through 4 were tested using the repeated measures two-way ANOVA. PCR data were analyzed using the one-way ANOVA. The Bonferroni test was used for post-ANOVA analysis. To assess the homogeneity of variance, Bartlett's and F-test were used. To assess the normality of the data, Kolmogorov-Smirnov test was used. A *p* value of less than 0.05 was considered statistically significant. Satatistics were analyzed using Prism software v. 4.03 (GraphPad Software, Inc., San Diego, CA, USA).

3.3. Results

3.3.1. Development of Acute Myocardial Injury

The administered 2 isoproterenol doses were effective in inducing myocardial injury and necrosis in post-AMI group. The development of the infarction was evident through three manifestations: 1) Electrocardiographic changes included significant J-point elevation on day 2 and R-wave amplitude reduction on days 2 through 4 (Figure 3-1 and 6), as well as the development of pathologic Q-wave in 50% of post-AMI rats; 2) Significant elevation of serum cardiac troponin I (cTnI), a specific marker for myocardial damage [248-250], in post-AMI rats by 170% (Table 1); 3) Presence of visible pale necrotic foci upon morphological examination of the isolated hearts. Mortality rate was 0/8 and 5/13 in controls and post-AMI rats, respectively. On days 1 and 2, heart rates of the animals in post-AMI group were significantly higher than controls (Day 1, 556±12 vs 396±4 bpm, Day 2, 571±7 vs 391±11 bpm in post-AMI and in controls, respectively). Moreover, PR-intervals on days 1 and 2 were not measurable in post-AMI group as they were masked by the increased heart rate and acute ECG changes. However, on day 4, just prior to verapamil administration, there was no significant difference between control and post-AMI groups with regards to PR-interval (40.4±4.4 vs. 37.6±1.6 msec, respectively) and HR (418±40 vs. 408±59 bpm, respectively).

3.3.2. Pharmacological Effects of Verapamil

Verapamil administration significantly prolonged PR interval and reduced heart rate in all animals (Figure 3-2). Myocardial injury, however, resulted in a

significant reduction in verapamil potency in prolonging PR-interval (Figure 3-2a and b). There was a significant 75% reduction in AUEC in the myocardial infarction rats as compared to the control animals. In post-AMI, the time-course-effect curves were generally lower than those in the control rats with the 120-min measurement being significantly different between the two groups (Figure 3-2a). Moreover, post-AMI rats were generally resistant to the heart rate reducing effects of verapamil (Figure 3-2c and d).

3.3.3. Verapamil Plasma Concentrations

As depicted in Table 3-1, plasma R-verapamil concentrations, observed 4.5 h post dosing, were elevated in post-AMI rats, however the change was not statistically significant. Similarly, plasma S-verapamil concentrations did not significantly differ between the control and the post-AMI groups.

3.3.4. Pro- and Anti-inflammatory Markers

A great inter-subject variability was noticed in all measured markers (Table 3-1). Despite its trend to be elevated in post-AMI group, concentration of the pro-inflammatory mediator, serum nitrite, was not significantly different from those observed in controls. TNF-α was only detectable in 4 animals (14.3±4.5 pg ml^{-1}) with high cTnI concentration (Figure 3-3b). IFN-γ was not detectable in either group. Serum cTnI was significantly correlated to AUEC of PR-interval prolongation produced by verapamil (Figure 3-3a). The relationship was non-linear and best described by $AUEC=Ae^{-0.22k(cTnI)}+123$ %*min. The change in

verapamil response was initially rapid but reached a plateau (123 %*min). The serum IL-4 concentration vs. maximum PR-interval prolongation plot revealed a significant direct correlation ($r = 0.8$, $p<0.05$). There was, however, a high degree of variability in this relationship so that when the two highest IL-4 concentrations were deleted the correlation became insignificant. Other relationships between response and mediators were also explored. However, due, likely, to the observed high variability none reached significance.

3.3.5. Western Blot of $Ca_v1.2$ Subunit of Cardiac L-type Calcium Channels

$Ca_v1.2$ protein density was significantly reduced in the myocardial infarction animals as compared with the control rats (Figure 3-4). Plotting $Ca_v1.2$ relative photometric density vs. verapamil response (AUEC of PR-interval prolongation) was suggestive of a significant direct correlation ($r = 0.6$, $p>0.05$), however, similar to what was observed for IL4 (section 3.3.4), the correlation was highly dependent on the highest $Ca_v1.2$ value which in its absence the significance vanished.

3.3.6. Real Time PCR of $Ca_v1.2$ mRNA of Cardiac L-type Calcium Channels

As depicted in Figure 3-5, $Ca_v1.2$ mRNA did not significantly change after infarction suggesting no change in $Ca_v1.2$ at the transcriptional level. The housekeeping gene GAPDH exhibited expression stability among the groups with no fold change difference between the groups.

Table 3-1. Effect of acute myocardial injury on the level of inflammatory markers and plasma verapamil concentration (n=8/group)

Group	**Control**	**Post-AMI**
cTnI (ng/ml)	4.6±0.9	12.7±2.6[a]
Nitrite (μM)	28.6±8.9	61.1±32.7
IL-4 (pg/ml)	19.9±9.1	9.7±4.4
Verapamil plasma concentration 4.5 h post-dose:		
S-verapamil (ng/ml)	35.8±0.8	26.6±5.6
R-Verapamil (ng/ml)	10.2±4.3	19.4±3.9

[a], significantly different from control ($p<0.05$)

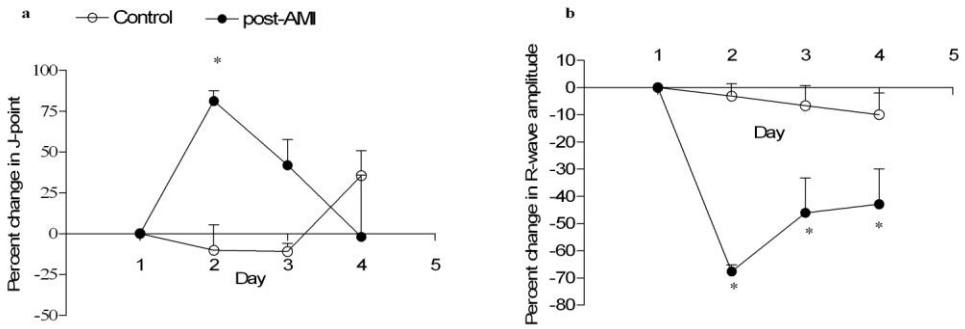


Figure 3-1. Electrocardiographic changes from Day 1 through 4 in control and post-AMI groups (n=8/group). (a) Percent change of J-point from baseline. (b) Percent change in R-wave amplitude from baseline. *, $p<0.05$ vs. Day 1 in the corresponding group.

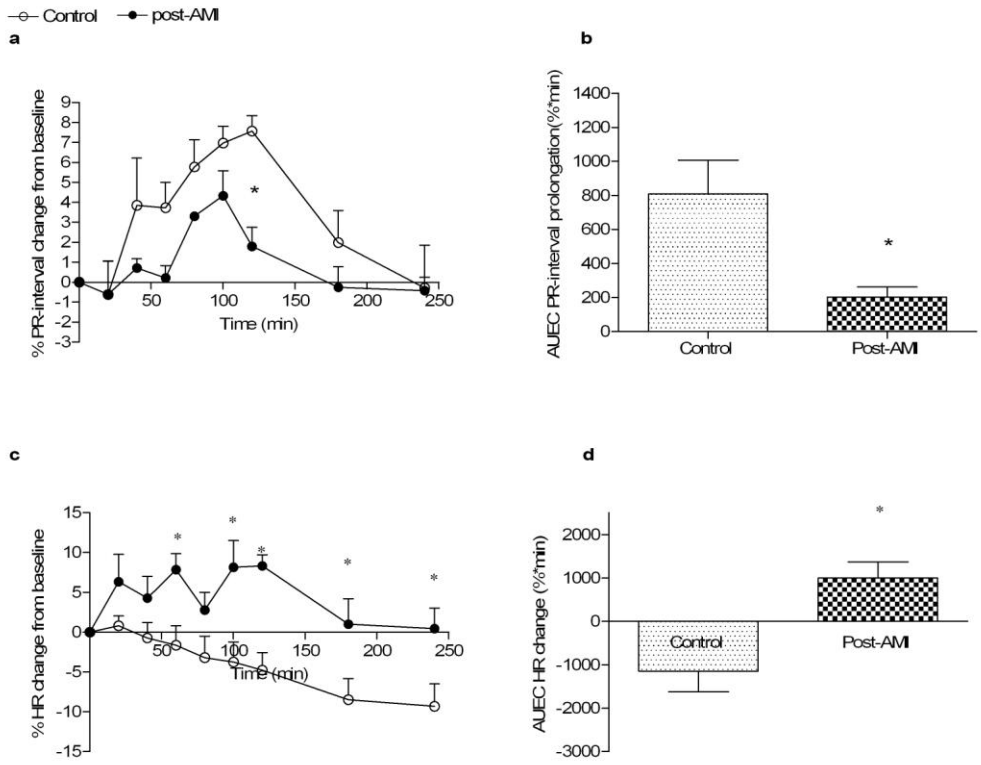


Figure 3-2. (a) Verapamil-induced PR interval prolongation in control and post-AMI rats (n=8/group) following a single oral 25 mg/kg dose of verapamil. (b) The area under the effect curve (AUEC) values derived from the data in panel a are shown. (c) Verapamil-induced heart rate (HR) change in control and post-AMI rats following a single oral 25 mg/kg dose of verapamil. (d) AUEC values derived from the data in panel c are shown. *, $p<0.05$ vs. control.

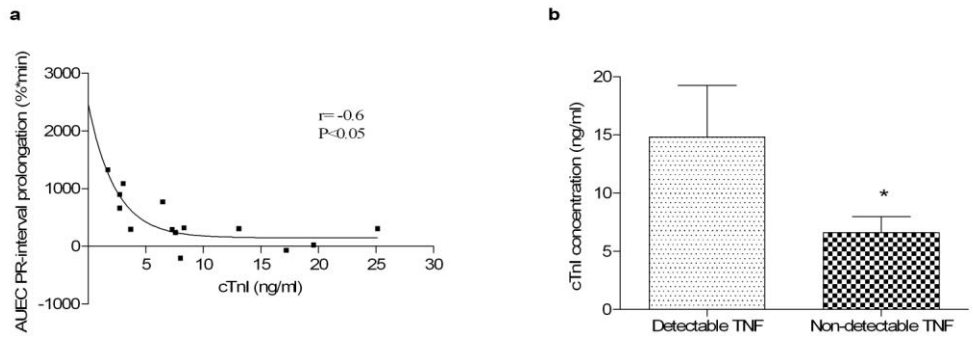


Figure 3-3. (a) Correlation between serum cardiac Troponin I (cTnI) and the area under the effect curve (AUEC) of PR-interval prolongation following a single oral 25 mg/kg dose of verapamil. (b) Serum cTnI concentration in rats with detectable and non-detectable tumor necrosis factor-α (TNF-α). *, $p<0.05$ vs. rats with detectable TNF-α. The line is the best estimate of the regression line. n=8/group.

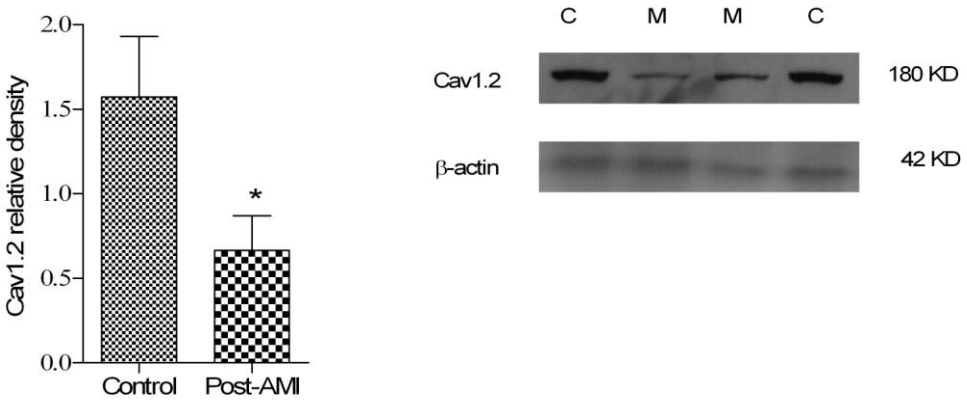


Figure 3-4. The effect of acute myocardial injury induction on cardiac $Ca_v1.2$ subunit density in rat hearts (n=8/group). Western blot of $Ca_v1.2$ subunit in control (C) and post-AMI (M) rat hearts. The bands density was measured using Image J software (National Institute of Health, Bethesda, MD, USA). *, $p< 0.05$ vs. control

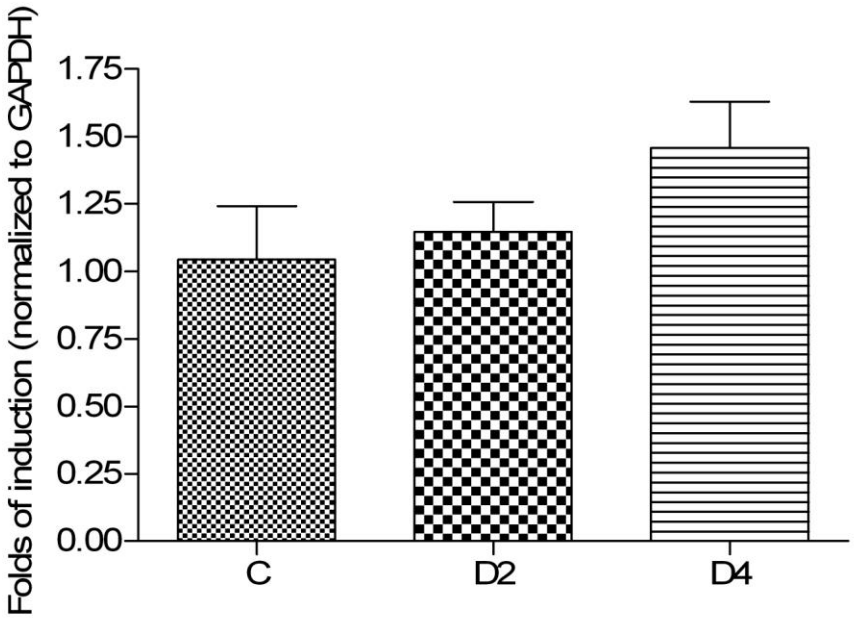


Figure 3-5. Real time reverse transcription polymerase chain reaction (RT-PCR) of $Ca_v1.2$ mRNA in control (C), day 2 (D2) and day 4 (D4) following induction of acute myocardial injury (n=4/group).

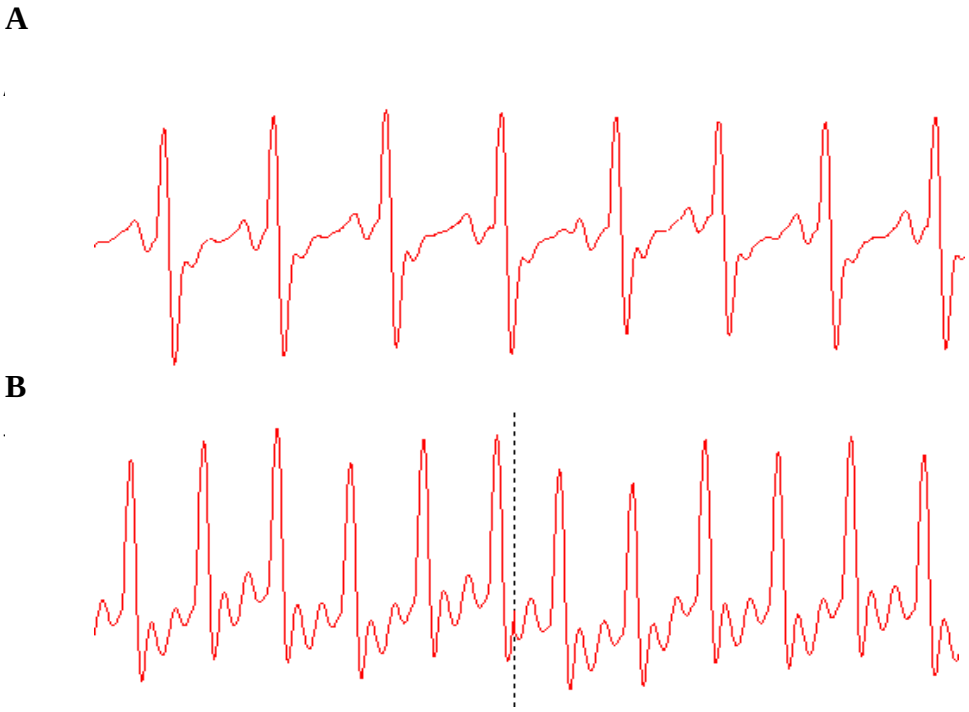


Figure 3-6. Electrocardiographic changes in post-AMI animals. A, Normal ECG trace at baseline. B, ECG trace of the same rat following administration of isoproterenol showing J point elevation.

3.4. Discussion

Isoproterenol-induced myocardial necrosis has been described earlier[241, 251]. The biochemical, electrocardiographic and histological features observed in this model has been found to resemble those observed in myocardial injury following myocardial infarction [240, 243, 244]. Isoproterenol produces myocardial necrosis by increasing myocardial oxygen consumption through increasing myocardial contractility and heart rate and by reducing myocardial oxygen supply through peripheral vasodilatation [241]. Electrocardiographic changes (Figure 3-1) combined with significant elevation of cTnI confirmed the development of myocardial injury. Our data, demonstrate, for the first time, that experimental myocardial tissue damage results in reduced response to verapamil, a calcium channel antagonist (Figure 3-2). The exact mechanism behind this observation is unclear. However, the present data clearly suggest a significant reduction in the calcium channel target protein (Figure 3-4) similar to what has been reported in adjuvant arthritis [9].

The effect of myocardial necrosis on verapamil potency is in agreement with what has been previously reported for other inflammatory conditions. Animal and human studies have shown that inflammatory conditions such as active rheumatoid arthritis [3, 193], aging [173] and obesity [1, 238] are associated with reduced pharmacodynamic response to calcium channel and β-adrenoceptor blockers [52]. Western immunoblot of $Ca_v1.2$ of the cardiac L-type calcium channels showed a significantly reduced amount of protein in post-AMI rats

(Figure 3-3). This may explain the mechanism of reduced verapamil efficacy in our model despite the fact that not all calcium channels are functional [252] and expression may reflect both the active and inactive channel forms. This is because it has been reported that inflammation-induced reduction in receptor density is associated with reduced binding to ^{3}H-nitrendipine, a dihydropyridine calcium channel blocker [9], and the number of dihydropyridine receptors are comparable to those of the functional L-type calcium channels in myocytes [253].

Pathogenesis and progression of cardiovascular diseases such as hypertension [78], congestive heart failure [12] and acute coronary syndromes [11, 20] have been attributed, in part, to inflammation. Myocardial damage following the infarction is an inflammatory process regulated by two classes of lymphocytes, the T-helper (Th) cells 1 and 2 [20]. Th1 secretes mainly pro-inflammatory mediators while Th2 largely secretes anti-inflammatory mediators [19]. Functional imbalance in Th1/Th2 ratio has been reported in AMI patients leading to the elevation of inflammatory mediators concentrations [20, 254]. The latter has been associated with poor prognosis and extended hospital stay [57, 58]. The present data, despite the strong and significant evidence we have presented for the emergence of myocardial injury and the reduced response to verapamil, do not equivocally suggest inflammation as the cause of our observations. Due likely to the observed great variability, the mean serum nitrite concentration presented herein was not significantly affected by AMI (Table 3-1). The observed variability was expected as it has also been reported for rheumatoid arthritis [3] and pre-adjuvant arthritis [9].

The variability is attributed to the disease severity that is beyond experimental control. Indeed, TNF-α was only detectable in the rats with elevated cTnI, a specific marker for myocardial damage [248-250]. cTnI, in turn, was significantly elevated in post-AMI animals (Table 3-1) and was negatively correlated with verapamil pharmacodynamic response (Figure 3-3a). In addition, there was a trend toward increased anti-inflammatory cytokine IL-4 concentration in the animals that demonstrated high response to verapamil. Although these changes suggest the involvement of inflammation and a link between the imbalance in Th1/Th2 ratio and response to verapamil in the rats receiving isoproterenol, we cannot rule out the possibility of contribution through other mechanisms. For example, a disruption of cellular proteins secondary to a reduction in the involved viable tissues due to infarction [239] may result in reduced verapamil potency.

Despite the observed downregulation of calcium channel target protein, the $Ca_v1.2$ mRNA did not significantly change as a result of myocardial injury (Figure 3-5). This suggests that inflammation-induced alterations of calcium channel target protein are at the post-transcriptional level. Further investigations are needed to confirm the mechanism and determine the functionality of the expressed calcium channels. It is also interesting to focus future efforts to investigate if our observation in whole heart can be extrapolated to the atria and AV node as the electrophysiological responses to verapamil are localized.

Inflammation has been shown to result in increased concentration of verapamil secondary to reduced clearance despite reduced potency [4, 8, 9, 91]. Here, we only observed a trend toward higher R-verapamil in Post-AMI group as compared with the control rats. This lack of significance in verapamil concentration between the two groups, however, should not be considered an equivocal evidence for a negative effect of myocardial injury on the clearance of verapamil. To eliminate the potential exacerbating effect of blood collection procedure on the pharmacodynamics outcome, we measure verapamil concentration only after the animals were euthanized at 4.5 h post-dose. At this time, the drug concentration has fallen to such low level that renders identification of the potential difference difficult [9].

Potency of verapamil is reduced in experimental myocardial injury in association with a reduction in the drug target protein, $Ca_v1.2$. This observation made in experimental cardiac injury may not unequivocally reflect what may be seen in humans. However, since the disease is associated with inflammation and the reduced response in noted in various type of humans and animals inflammatory conditions, our observation may prompt efforts to investigate reduced drug response as a potential contributory factor in poor therapeutic outcome in post myocardial infarction patients.

Chapter 4

4. Effect of obesity on response to cardiovascular drugs in pediatric patients with renal disease[‡]

4.1. Introduction

The incidence of childhood obesity in North America has increased dramatically over the past three decades[60-63]. Obesity is linked to poor disease outcome in renal transplant patients[65] and increased propensity to some adult conditions like cardiovascular diseases[64]. In addition, obesity has been regarded as an inflammatory process[13, 66] complicated with chronic low grade inflammation[13, 14]. Overweight and obese children have elevated inflammatory mediator levels such C-reactive protein (CRP) and tumor necrosis factor α (TNF-α) as compared to the general children population[71-73]. Adult obesity and other inflammatory conditions associated with elevated concentrations of pro-inflammatory mediators such as rheumatoid arthritis have been found to be linked to reduced response to calcium channel blockers (CCBs) such as verapamil[1, 3, 4, 9]. This has been attributed to inflammation-induced reduction in binding to L-type calcium channels[4, 9] and downregulation of calcium channel target protein[9]. This effect of inflammation is important because obesity and other inflammatory states complicate many diseases like hypertension, angina and acute myocardial infarction, that may

[‡] A version of this chapter has been published. Hanafy S, Pinsk M and Jamali F. Pediatric Nephrology 2009; 24 (4): 815-821

require the use of calcium channel blockers[255-257] contributing to poor disease outcome[58].

Pediatric patients with renal disease have an increased propensity to hypertension compared to children with normal renal function[257]. This has been attributed to many reasons such as activation of the renin-angiotensin-aldosterone system, fluid overload and corticosteroid therapy[257]. CCBs such as nifedipine and amlodipine and angiotensin converting enzyme inhibitors such as ramipril and enalapril are the most commonly prescribed drugs to treat hypertension in children with renal disease[219-221]. The effect of obesity on the pharmacological response of these drugs in children with renal disease has not been studied before.

The purpose of the study is to investigate the effect of obesity on the pharmacodynamic response to calcium channel and angiotensin II blockers in pediatric patients with renal disease who are currently prescribed these classes of drugs.

4.2. Patients and Methods

A retrospective chart review study was conducted on health records of 263 pediatric patients treated for renal disease at the Pediatric Nephrology Outpatient Clinic at University of Alberta Hospital/Stollery Children's Hospital between 2002 and 2006. The study was approved by The Health Research Ethics Board at University of Alberta. Patient medical records were chosen based on the

International Classification of Diseases, 9th edition (ICD-9) codes for renal conditions in which antihypertensive therapy are routinely employed [258] (Table 4-1). Patients were allocated to a diagnosis category based on the identification of coding priority. Figure 4-1 depicts the study design. Charts were reviewed to identify whether antihypertensive medications were prescribed to these patients. Inclusion criteria were males or females younger than 18 years; patients treated with CCBs (nifedipine or amlodipine) or angiotensin interrupting agents (ANGI, angiotensin converting enzyme inhibitors, i.e., captopril, ramipril, enalapril and angiotensin II receptor blockers, i.e., candesartan, losartan, and valsartan) alone or the combination of the two; patient's chart with sufficient demographic data and blood pressure measurements were chosen.

Data collected were age, sex, weight, height, body mass index (BMI), drug treatment, dosage and pre- and post-therapy blood pressure measures. Weight, height and BMI percentiles were determined for each patient using specific nomograms for the corresponding age and sex[259]. Blood pressure was measured according to the recommendations of the fourth report on the diagnosis, evaluation, and treatment of high blood pressure in children and adolescents [136]. It was measured by physicians and/or nurses using manual/auscultatory methods. All the measures were done in the hospital. Systolic and diastolic blood pressure 95th percentile values for the corresponding age, sex and height were also determined for each patient using 2004 guidelines[136]. Systolic percentiles for patients younger than 1 year of age were determined using the 1987 guidelines [260].

Patients were considered hypertensive if their blood pressure values were equal to or greater than the 95th percentile blood pressure (BP) for the corresponding age, sex and height[136]. Patients' characteristics depicted in Table 4-2 were compared between the obese and non-obese patients using the two-tailed Student's t-test

An obese patient aged between 2-18 years was defined as the one with body mass index (BMI) value equals to or greater than the 95th percentile for healthy children with corresponding age and sex[64]. Patients younger than 2 years of age whose weight percentile are more than or equal the 95th percentile of corresponding age and sex were considered obese.

The influence of obesity on the systolic and diastolic blood pressure lowering effects of calcium channel blockers and ANGI was determined. This was achieved by measuring two outcome variables. First, categorizing patients as responders versus non-responders based on at least 10% reduction from the baseline systolic and/or diastolic blood pressure. The effect of obesity was tested using Multivariate Logistic Regression [261]. Second, the percent reduction in the blood pressure from the baseline was measured and compared between obese and non-obese using the two-tailed Student's t-test. The effect of other covariates i.e., age, sex, diagnosis and, corticosteroid therapy on drug response was also tested using Multivariate Logistic Regression. The *p*-value was set at <0.05. Statistical analyses were carried out using Statistical Analysis System (SAS) software version 9.1 (SAS Institute, Cary, NC, USA).

Table 4-1. ICD-9 (International Classification of Diseases, 9th edition) diagnostic codes used

Coding Priority	ICD-9 code	Diagnosis	Number of patients included
1	585	Chronic renal failure	5
	593.X	Chronic renal insufficiency	
2	580.X	Acute glomerulonephritis	8
	583.X	All glomerulonephritis	
	599.7	Hematuria	
	287.0	Henoch Schönlein purpura (glomerulonephritis)	
	582.X	Chronic Glomerulonephritis	
3	581.X	Focal segmental glomerulonephritis and nephrotic syndrome	23
4	593.X	Reflux nephropathy	3
	596.X	Bladder dysfunction	
	753.X	Urologic abnormalities	
	589.X	Urologic abnormalities	
5	401.9	Hypertension	9
Total			48

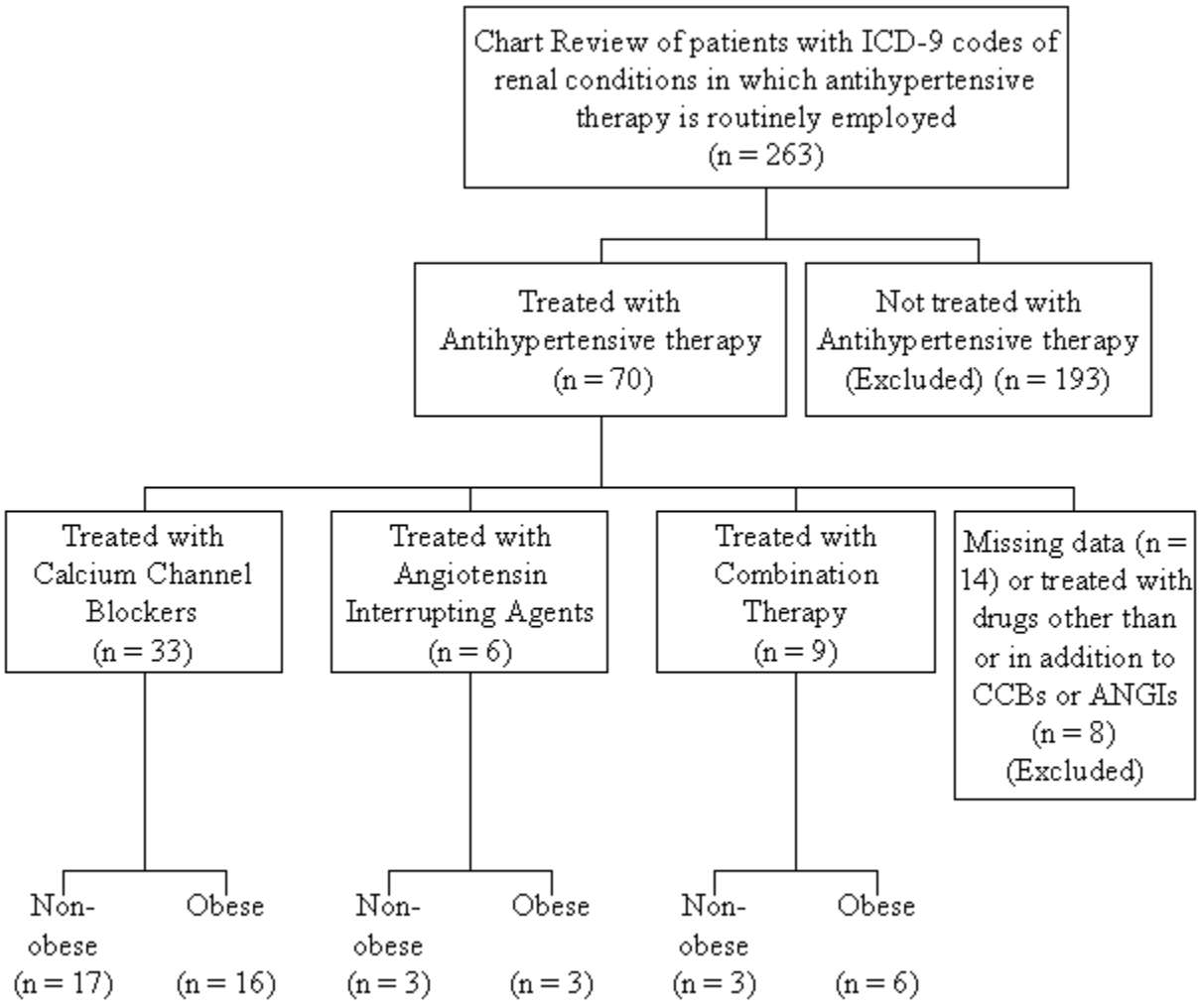


Figure 4-1. Flow chart of patients recruitment and grouping strategy.

4.3. Results

A total of 263 patients' medical records were reviewed. The highest number of patients on antihypertensive therapy was those who, according to ICD-9 diagnostic list, had been placed under hypertension (100%) followed by nephrotic syndrome (65%) categories. Patients with urologic abnormalities had the lowest percentage of those on antihypertensive therapy (5%). Overall, 70 patients were found to be treated with antihypertensive drugs: Chronic renal failure (n = 6), glomerulonephritis and related conditions (n = 11), nephrotic syndrome (n = 35), urologic abnormalities (n = 6) and hypertension (n = 12).

Six patients were excluded because they were missing height values needed for determining BMI and BP percentiles. Another 8 patients were excluded because they were treated by antihypertensive medications other than or in addition to CCBs and ANGIs. Of the latter eight excluded patients, five were obese. They had been treated with multiple medications because they were resistant to therapy. Another eight patients were on antihypertensive medications before assessment by the clinical service and so they were excluded from the study. Consequently, a total of 48 patients were included in the study (Table 4-2). Age and height distributions were not significantly different between obese and non-obese patients. There were significantly more obese males than females ($p<0.05$). Twenty five patients were on corticosteroid therapy.

Thirty three patients (17 non-obese and 16 obese) were treated with calcium channel blockers (amlodipine, nifedipine XL or regular acting nifedipine). Six patients were treated with ANGIs (captopril, ramipril, enalapril and losartan) and nine patients were treated with a combination of an ANGI and a CCB. There was no significant difference in CCBs and ANGI dosages between obese and non-obese patients (Table 4-3).

The systolic response to the calcium channel antagonist therapy, measured as at least 10% reduction from the baseline, was significantly less in obese (12.5%) as compared with the non-obese (52.9%) group. With regards to the diastolic response although numerically different (58.8 and 25% for non-obese and obese, respectively) no significance was reached. With regards to the percent reduction in systolic and diastolic blood pressure, the influence of the calcium channel antagonists was significantly less in obese as compared with the non-obese patients (Figure 4-2). Among the examined covariates, only obesity and corticosteroid therapy had significant influence on the systolic and diastolic response to calcium channel antagonists, respectively (Table 4-4). Corticosteroid treated patients had a significantly reduced diastolic response (25%) to CCBs as compared to non-treated ones (69.2%). None of the tested covariates, including obesity, was found to significantly influence the response to ANGIs. As depicted in Figure 4-2, the percent reduction in systolic and diastolic blood pressure, the influence of ANGIs, alone or in combinations with CCBs, was not significantly different in obese as compared with the non-obese patients (Figure 4-2).

Table 4-2. Patients' characteristics

	Non-obese	**Obese**
# (%)	23 (48)	25 (52)
Age, y	8.5 ± 0.7	8.3 ± 1.1
range, y	2.58-15	0.17-16
Sex, #(%)		
Males	12 (52.17)	21 (84)*
Females	11 (47.83)	4 (16)
Height, cm	131±4.2	124.4±7.5
range, cm	92-169.4	48-180
Height percentile	57.6±7.3	54.2±6.9
Weight, kg	32.5±3.0	47.1±6.3*
range, kg	12-61.3	6.6-114.8
Weight Percentile	59.6±6.6	93±1.1*
BMI, kg/m^2	17.9±0.7	26.5±1.2*
range, kg/m^2	12.4-24.8	18.6-37.1
BMI percentile	61.3±6.1	95±0*

*, $p<0.05$ from non-obese.

Table 4-3. Mean calcium channel blockers (CCB) and angiotensin interrupting agents (ANGI) doses (mg/m2/d)

	Non-obese	Obese	p-value
Amlodipine			
#	7	2	
dose (mg/m2/d)	6.9±1.2	8.3±2.5	0.60
Nifedipine (short acting)			
#	11	13	
dose (mg/m2/d)	10.7±2.4	9.6±1.9	0.73
Nifedipine (long acting)			
#	2	7	
dose (mg/m2/d)	77.2±34	56±15.8	0.56
Ramipril			
#	4	4	
dose (mg/m2/d)	7.7±1.0	5.6±1.9	0.36
Other ANGI (enalapril comparative dose) [136, 262]			
#	2	5	
dose (mg/m2/d)	4.9±3.7	5.0±0.9	0.97

Table 4-4. Adjusted odds ratios, 95 % confidence intervals (CI) and p-values of different variables tested for their association with systolic and diastolic responses to calcium channel blockers.

Co-variate	**Systolic Response**			**Diastolic Response**		
	Odds ratio	95 % CI	*p*	Odds ratio	95 % CI	*p*
Age	0.98	0.79-1.2	0.85	1.05	0.86-1.3	0.66
Sex (females vs. males)	0.36	0.04-3.4	0.38	0.88	0.1-7.5	0.9
Obesity (non-obese vs. obese)	12.26	1.2-122	0.03	3.93	0.6-25.9	0.16
Nephrotic Syndrome (non nephrotic vs. nephrotic)	5.66	0.78-41	0.09	0.17	0.01-1.8	0.14
Corticosteroid therapy (not treated vs treated)	1.83	0.3-11.7	0.53	15.1	1.3-176	0.03

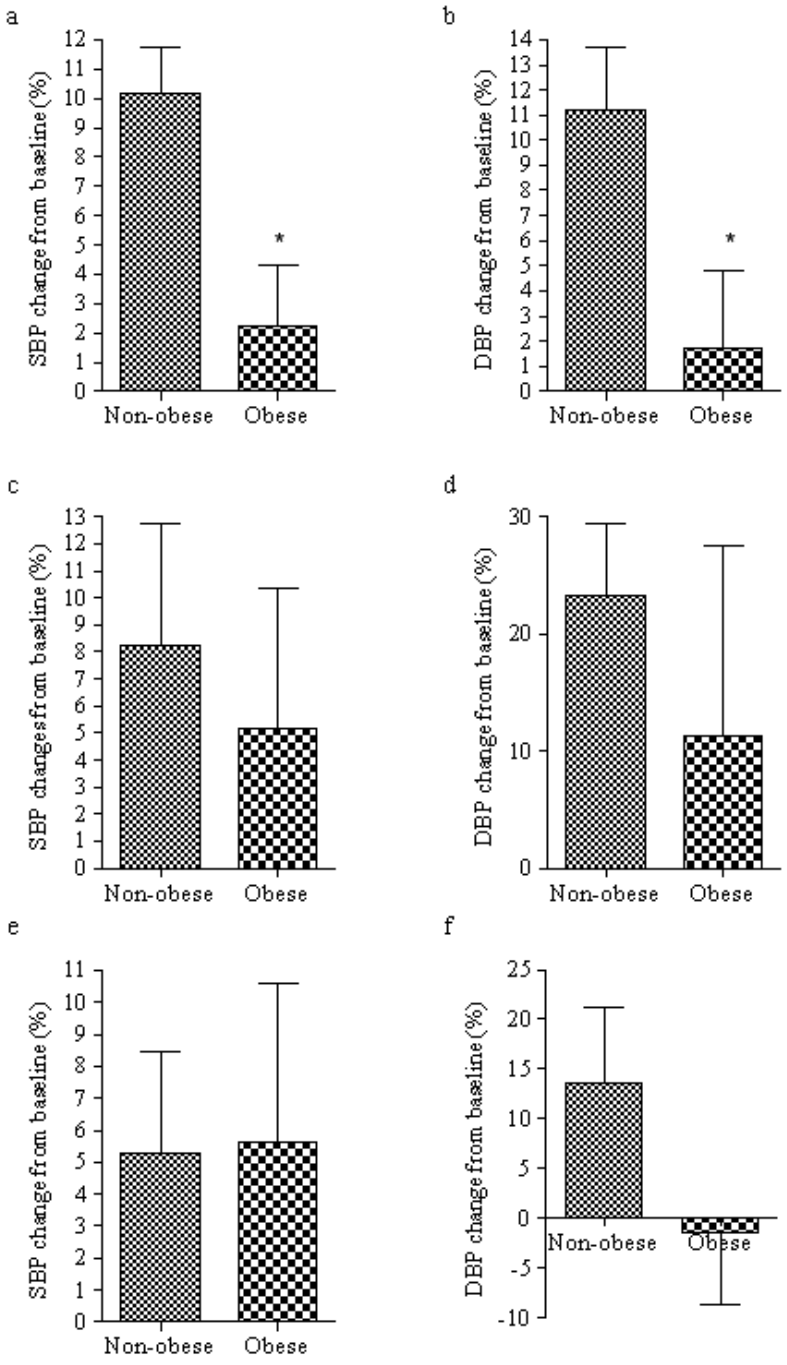


Figure 4-2. Percent changes of systolic (SBP) and diastolic (DBP) blood pressure from baseline in obese and non-obese patients treated with calcium channel blockers (a, b), angiotensin interrupting agents (c, d) or combination of the two (e, f). *, $p<0.05$ from non-obese.

4.4. Discussion

In adults, rheumatoid arthritis[3], old age[2] and obesity[1] result in reduced response to the calcium channel antagonist verapamil. These conditions are considered chronic low grade inflammatory states and are all associated with elevated circulating concentration of pro-inflammatory mediators such as CRP and TNFα[13, 14]. Accumulation of adipose tissue in obese individuals has been linked to increased macrophage infiltration and, chemokine and cytokine production[66]. Obese children also have elevated serum concentrations of CRP[71-73] and TNF-α[73]. It is therefore important to explore if obese children also exhibit lower response to calcium channel therapy as compared to non-obese population. Our data indicate that systolic blood pressure in obese children is less responsive to CCBs than non-obese children (Figure 4-2, Table 4-4). Since the patients were dosed based on their BSA rather than their body weight and there was no significant difference in dosages, the possibility of under-dosing can be ruled out. The exact mechanism behind this observation is unknown. Data from animal studies indicate that the reduced response to calcium channel blockers in inflammatory conditions is due to downregulation of L-type calcium channels secondary to a reduced drug receptor binding[4, 9], that by itself, is caused by depressed target protein expression[9].

All pediatric patients chosen in this study had underlying renal complications (Table 4-1). Explanations put forward for the high incidence of elevated blood pressure in pediatric patients with renal disease include activation

of the renin-angiotensin-aldosterone system, fluid overload and corticosteroid therapy[257]. These patients are usually treated with antihypertensive drugs such as CCBs and ANGIs. Considering the fact that out of the 263 patients reviewed in this study, 70 (27%) needed antihypertensive therapy, and out of these 50% were obese, our observation becomes important. Hypertension by itself is an inflammatory condition so that hypertensive obese children have been found to have higher concentrations of inflammatory mediators than non-hypertensive ones[263]. The magnitude of the reduced response observed in our study, therefore, may be due to combination of obesity and hypertension. Indeed, we observed the downregulating effect of obesity by including only a relatively small population size in the study.

Obesity was not the only confounding variable; corticosteroid therapy was also associated with reduced diastolic response to CCBs. Prednisone and methylprednisone are commonly prescribed drugs in controlling the progression of some kidney diseases or as immunosuppressants in renal transplant recipients [217, 226]. Corticosteroids are known to antagonize the antihypertensive effects of drugs[257]. This effect has been explained by three mechanisms: Induction of positive sodium balance with consequent water retention, enhancement of the sensitivity of tissues to catecholamines with subsequent vasoconstriction and central nervous system activation[264, 265].

Patients diagnosed with hypertension-related nephrotic syndrome had the highest requirement of antihypertensive medications. This is expected because of the nature of the disease and concomitant treatment with corticosteroids[255, 266]. On the other hand, patients with urologic abnormalities had the lowest requirements of antihypertensive treatment. Thus, knowing the underlying kidney disease and its management can help to predict drug response. Nephrotic syndrome patients were more prone to reduced efficacy of antihypertensive medications, possibly explained by hypoalbuminemia-induced water retention, obesity and corticosteroid therapy[255, 257]. On the other hand hypertensive patients with urologic abnormalities are more likely to respond to medications because they do not have similar risk factors for resistance to therapy.

Although sex was not significantly associated with reduced drug response, there were more obese males than females. This may explain in part the previous observation that the long term antihypertensive efficacy of amlodipine in pediatric patients with chronic kidney disease is significantly higher in females than males[267].

Reduced response to drugs, including calcium channel blockers, in inflammation-complicated conditions may contribute to poor disease prognosis and therapy outcome. Other cardiovascular diseases such as acute myocardial infarction and heart failure are also associated with elevated proinflammatory mediator concentration[11, 12]. Indeed, morbidity and mortality in these patients are

correlated with the magnitude of these elevations[58]. The potential contribution of receptor downregulation in the poor therapy outcome in these patients cannot be ruled out.

Due to the retrospective nature of the study we did not have access to plasma concentration of the antihypertensive drugs, hence, cannot equivocally rule out a potential contribution to our observation of an altered pharmacokinetics. This is important in light of the potential effect of hypoalbuminemia observed in our patients. Hypoalbuminemia may cause reduced plasma protein binding of drugs resulting in a potential increase in concentration at the site of action[268, 269] This reduced protein binding may also give rise to accelerated overall clearance of the drug. The two potential mechanisms often offset each other and render the influence of hypoalbuminemia on the pharmacokinetics negligible[268-270].

It is not well known if our observation can be extrapolated to the action of other cardiovascular drug in humans. It is known that β-adrenergic receptors are also downregulated in animal models of inflammation[52]. In contrast, our present data indicate that obesity had no significant influence on the potency of angiotensin interrupting agents. This is in agreement to what has been found in adults: inflammation does not alter the pharmacological response to angiotensin II receptor blockers such as valsartan[221] and losartan[222]. This attributed to the suppression of angiotensin II that is a potent pro-inflammatory mediator[202]. In addition, these drugs have been found to improve proteinuria in some kidney

diseases such as nephrotic syndrome and acute glomerulonephritis [271], an added advantage for their use.

The limitations of the present study include its retrospective nature and the relatively small population size. Nevertheless, the influence of obesity of response to CCBs appears to be of the magnitude that was evident even using a small sample size and the variability inherent in this type of studies. The disease severity, degree of obesity and inconsistency in corticosteroid dosing, might have further contributed to the variability in drug response. A larger prospective clinical trial, where drug therapy and response measures are standardized, should be instituted to confirm the results obtained from the present study.

In conclusion, obesity and corticosteroid therapy are important confounding factors that govern the responsiveness of pediatric patients treated for renal disease with antihypertensive drugs. Obesity should be considered when initiating antihypertensive drug therapy for children with kidney disease.

Chapter 5

5. Differential expression of housekeeping genes in rat model of pre-adjuvant arthritis

5.1. Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by autoimmune joint inflammation and extrarticular complications. The latter includes cardiovascular diseases, rheumatoid nodules, ocular inflammation, pulmonary diseases, neuropathy, splenomegaly and thyroid hormone alterations.[18] The degree of systemic inflammation is considered as one of the main factors contributing to these adverse cardiovascular events[18, 42]. To study the effect of inflammation on disease pathogenesis and drug targets at the molecular levels, several experimental techniques are used, one of which is real time reverse transcriptase polymerase chain reaction (RT-PCR).

Real time RT-PCR is a facile and efficient technique used to detect and quantify expression of target genes. Determination of the target gene expression using RT-PCR requires normalization to housekeeping (HK) genes and calculation of fold changes using the delta delta C_T ($2^{-\Delta\Delta C_T}$) method[247]. Generally, housekeeping genes are constitutively expressed in the cells and measured simultaneously with the target gene of interest to account for inter-sample variability. The suitability of housekeeping genes under different conditions has

been thoroughly discussed in literature[272-274]. It has been found that the expression of some of the most commonly used HK genes can vary in different disease states, age groups, species, organs or experimental treatments[275-284].

Generally, there are three criteria that determine the best choice of housekeeping genes. First, they should be constitutively expressed in the tissues and/or cells under investigation. Second, they should exhibit high expression stability as determined using available software such as ge-NORM[285] and NormFinder[286]. Third, their expression should not be affected by the diseases or pathological conditions.

In studies involving inflammatory models, several housekeeping genes are used such as glyceraldehyde-3-phosphate-dehydrogenase (GAPDH), β-actin and 18s ribosomal RNA[118, 276, 287, 288]. The effect of inflammation on the expression profile of HK genes has not been thoroughly studied,[276] and to the best of our knowledge, validation of suitable housekeeping genes in chronic inflammation has not been reported. The objective of the present study is to determine the effect of inflammation on the expression profile of the three commonly used HK genes. We used the rat model of pre-adjuvant arthritis (PRE-AA) a model that is commonly used as a model for the human rheumatoid arthritis in terms of development of systemic inflammation and arthritis[289]. We tested the expression profile of HK genes in rat liver, heart, kidney and intestine.

5.2. Methods

5.2.1. Experimental Animals

The study protocol was approved by the Health Sciences Animal Policy and Welfare Committee of the University of Alberta. The experiments were carried out on male Sprague-Dawley rats (220-280 g). They were housed in a controlled-temperature room with a 12-hours dark/light cycles.

5.2.2. Experimental Protocol

Two animal groups (n=4/group) were used, pre-adjuvant arthritis (Pre-AA) and healthy control (Control). The PRE-AA group received 0.2 ml of 50 mg/ml *Mycobacterium butyricum* suspended in squalene into the tail base as intra-lymphatic injections. Control animals received an equal volume of normal saline into the tail base. On day 12, rats were euthanized while under halothane/oxygen anesthesia by exsanguination and their organs (heart, liver, kidney and the upper segment of the small intestine) were excised and frozen instantly in liquid nitrogen. The excised organs were stored in -80 degrees until analyzed.

5.2.3. Real Time Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated from the frozen organs using TRIzol reagent (Invitrogen, Carlsband, CA, USA) according to the manufacturer's instructions (Chapter 3). This was followed by spectrophotometric quantitation of the isolated RNA at 260 nm. Moreover, the 260/280 ratio was also measured to check the purity of the samples. cDNA was synthesized from 1.5 μg total RNA samples with the random

primers scheme using the high capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA). Real Time PCR was performed on an ABI 7500 Real Time PCR system (Applied Biosystems, Foster City, CA, USA). Forward and reverse primers used for the experiments are shown in Table 5-1. Primers were purchased from Integrated DNA Technologies (Coralville, IA, USA). The housekeeping genes tested were rat GAPDH, β-actin and 18s rRNA. PCR products were produced and detected quantitatively using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA). Melting curves were carried out to confirm amplification of single sequences and absence of primer dimers. Each RNA sample was repeated three times on three different occasions. Cycle threshold (C_T) for each sample was determined and the average C_T was estimated for each Control and PRE-AA rats (n=4/group) and were compared to one another. Fold changes were calculated for each occasion (n=3) using the delta C_T method ($2^{-\Delta C_T}$), where $\Delta C_T = C_{TAA} - C_{TControl}$[247, 275]. Average fold changes were calculated. Statistical significance between average C_T of the Control and PRE-AA groups and the fold of changes in PRE-AA rats from baseline were analyzed using a two-tailed student t-test. Statistical tests were done on linearly transformed values using 2^{-C_T} formula[247]. The homogeneity of variance was determined using the F-test. Data are expressed as mean ± SD. A *p* value of less than 0.05 was considered statistically significant. Satatistics were analyzed using Prism software v. 4.03 (GraphPad Software, Inc., San Diego, CA, USA). The expression stability of the genes was determined using geNORM and NormFinder programs. geNORM is a visual basic application for Microsoft Excel that is used

to estimate the expression stability (M) of various genes through comparing the mean pairwise variation of the gene with all other genes[285]. To determine the best housekeeping gene based on intra- and inter-group variability of gene expression, NormFinder (Microsoft Excel add-in) was used;[286] the lower the M values determined by geNORM and stability values determined by NormFinder, the higher the expression stability[285, 286].

5.3. Results

Table 5-2 depicts the average C_T values for the tested housekeeping genes in the examined rat organs. Plotting the average fold of change of GAPDH, β-actin and 18s rRNA in Pre-AA animals (Figure 5-1) revealed that there are organ-specific alterations in gene expression. While β-actin expression minimally changed in the heart, it was significantly reduced by 52, 62 and 81% in the liver, kidney and intestine of Pre-AA rats, respectively. GAPDH expression was also significantly reduced by 61% in the intestine of Pre-AA rats as compared to Control. 18s rRNA expression was the least altered gene in all the examined organs. Analysis of the data using geNORM and NormFinder resulted in observations similar to the abovementioned fold of changes (Figure 5-1). The estimated average expression stabilities (M) as determined by geNORM for GAPDH (liver, 0.974; kidney, 0.544; heart, 0.334; intestine, 1.884) and 18sRNA (liver, 0.846; kidney, 0.648; heart, 0.363; intestine, 2.246) were generally lower than those for β-actin (liver, 1.423; kidney, 0.745; heart, 0.368; intestine, 2.449). In other words, GAPDH and 18s RNA exhibited more expression stability than β-actin. Similarly, NormFinder

estimated that GAPDH and 18sRNA have more expression stabilities and less inter- and intra-group variabilities than β-actin. The estimated stability values as determined by NormFinder for GAPDH (liver, 0.271; kidney, 0.189; heart, 0.046; intestine, 0.352) and 18sRNA (liver, 0.161; kidney, 0.334; heart, 0.100; intestine, 0.54) were lower than those for β-actin (liver, 0.476; kidney, 0.384; heart, 0.101; intestine, 0.732). Interestingly, although GAPDH fold of change in Pre-AA animals were more apparent in the rat intestine, its M and stability values, as determined by geNORM and NormFinder, respectively are lower than 18s rRNA.

Table 5-1. List of primers used in RT-PCR

Gene	Forward primer	Reverse primer	Ref
GAPDH	GCACCACCAACTGCTTAGC C	CTGAGTGGCAGTGATGGCA T	[33]
β Actin	CCAGATCATGTTTGAGACC TTCAA	GTGGTACGACCAGAGGCAT ACA	[34]
18s rRNA	GGGAGGTAGTGACGAAAA ATAACAAT	TTGCCCTCCAATGGATCCT	[290]

Table 5-2. Average C_T values for housekeeping genes in different organs in Control and pre-Adjuvant Arthritis (pre-AA) rats (n=4/group).

Organ	Housekeeping gene	Control	AA
Liver			
	GAPDH	15.04±0.44	14.99±0.63
	β-Actin	21.15±1.39	22.23±0.68
	18s rRNA	10.36±0.26	10.31±0.18
Kidney	GAPDH	14.98±0.37	15.30±0.15
	β-Actin	17.81±0.54	19.19±0.28*
	18s rRNA	10.81±0.28	10.99±0.48
Heart	GAPDH	13.95±0.84	13.71±0.13
	β-Actin	17.48±0.88	17.56±0.12
	18s rRNA	10.73±0.43	10.74±0.24
Intestine	GAPDH	18.70±1.79	20.07±1.52
	β-Actin	21.82±3.66	24.24±2.36
	18s rRNA	10.34±0.66	10.53±0.63

Data a represented by mean±SD. *, $p<0.05$ vs Control rats

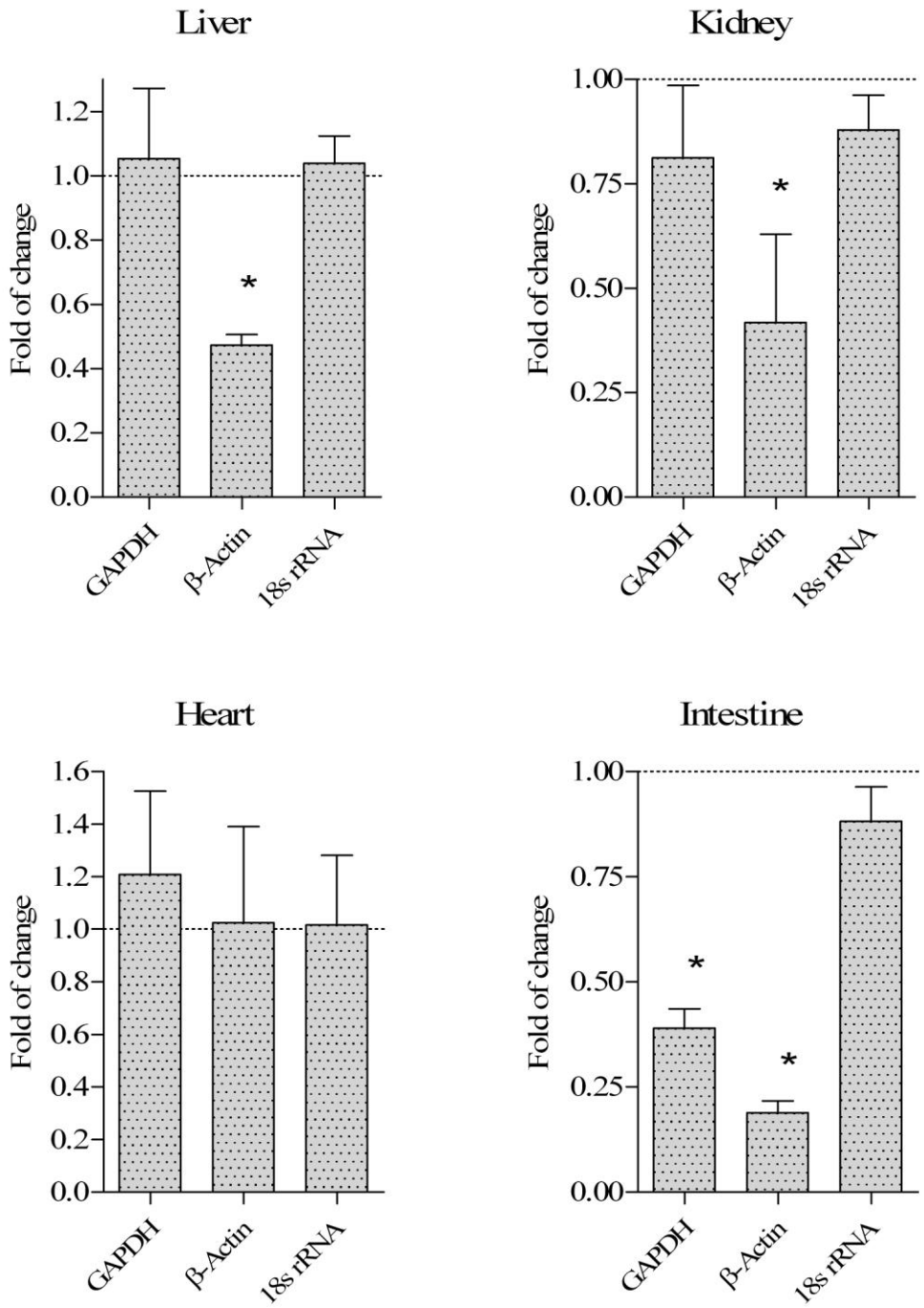


Figure 5-1. Average fold of changes (n=3/group) in GAPDH, β-actin anf 18s rRNA in Pre-AA rats compared to Control (dotted line) in different organs. *, $p<0.05$ vs baseline expression in Control rats.

5.4. Discussion

The present examination of the suitability of using three housekeeping genes in RT-PCR in a chronic inflammatory model revealed, for the first time, that the choice of a housekeeping gene depends on the organ examined. In experiments testing the relative expression of target genes in the liver and kidney, we suggest using 18s rRNA or GAPDH. In experiments using rat intestine we suggest using 18s rRNA. Any of the three tested genes can be used as HK in the heart because they have close variability and stability values.

GAPDH mRNA, which is constitutively expressed in most tissues and cell types, encodes for an important enzyme in carbohydrates metabolism that plays a key role in glycolysis[291]. It has been used as an experimental internal control for decades. However there have been many reports showing that GAPDH gene expression may be altered in different diseases, conditions and some experimental treatments[276, 292-294]. β-actin mRNA codes for one of the most widely expressed proteins in living cells. It is an integral part in the cell cytoskeleton. Like GAPDH, it is used for decades in RNA experiments such as Northern blots and RT-PCR. Few studies have questioned its use as an internal control due to its variability[276, 294, 295]. The ribosomal RNA, 18s rRNA, is an important constituent of the ribosome, the protein synthesizing machinery in the cell. 18s rRNA exhibits a relatively stable and low variability expression profile in all tissues[296]. Therefore, its use as a housekeeping gene is usually preferred in RT-PCR experiments. However, being highly expressed in tissues, 18s rRNA requires

careful titration of its primer concentrations to provide a practical expression signal without quickly consuming the reaction components[297].

18s rRNA was found to be the gene with the lowest fold change difference among samples in the liver, kidney, heart and intestine (Figure 5-1). GAPDH was the second gene in the liver, kidney and heart. However, in the intestine its expression was reduced by 61% affecting its suitability to be used an internal control in this tissue. On the other hand β-actin was the gene with the highest difference between groups except in the heart. Despite the observed fold changes of GAPDH and β-actin in the examined rat organs, average C_{Ts} were not statistically significant between Control and Pre-AA rats (Table 5-2) except β-actin in the rat kidney. This is because the use of raw C_T in comparisons poorly represents fold changes and the use of $2^{-\Delta C_T}$ method, to determine fold changes, has been described as an appropriate method to determine the best housekeeping gene to be used as an internal control[247, 275]. Using geNorm and Normfinder, we found that the top 2 genes were GAPDH and 18s rRNA. However, in program analysis, GAPDH preceded 18s RNA in stability ranking. The reason for this discrepancy comes from the nature of the algorithms followed by both programs. geNORM considers instability when the pattern of the variation of the gene differs from other genes. For example, in the intestine as both β-actin and GAPDH are downregulated and 18s RNA is not, this skews the stability estimation towards the former two genes. NormFinder estimates the stability values based on the inter- and intra-group variations. In addition it assumes that

the average variation among groups of all genes is similar. So if a gene deviates from the average pattern, as in the case of 18s rRNA in intestine it is assigned a lower stability ranking than GAPDH. These discrepancies have been previously reported[298, 299].

The findings of the present study are of importance in RT-PCR studies carried out in inflammatory diseases. Inflammation is the host's response to infection, injury or irritation. The development of the inflammatory response is a complex process that involves increased expression of a remarkable amount of chemicals such as cytokines and chemokines in a paracrine, autocrine, and/or endocrine fashion that enhances host's defense through immune cells recruitment and activation, antibodies production and complement system activation[17]. This has been found to be associated with altered expression of other genes either as a part of the host's defense mechanism or due to shifting priorities in the cellular expression machinery. Examples of the affected genes are metabolizing enzymes,[37, 96, 97] transporters,[113] receptors[4, 5, 7, 9, 38] and plasma proteins[90, 91, 177]. Therefore, in order to determine the effect of inflammation on disease pathogenesis and prognosis we need to have robust experimental techniques that allow proper interpretation of the results.

The observed changes in β-actin gene expression in different rat organs can be reflected at the translational level by downregulation of the protein. Similar to RT-PCR, β-actin has been used as a loading control in Western blot

(WB) experiments to account for inter-sample variability. There is a debate in literature regarding the suitability of the use of β-actin in WB. While some researchers questioned its use as an internal loading control in some diseases,[300, 301] others have recommended its use because of its expression stability[302, 303]. The expression stability of β-actin gene observed in the rat heart is in parallel to what we have found in WB experiments done in our lab. β-actin protein expression did not change among the groups in the heart of rat model of pre-adjuvant arthritis.[9] The effect of inflammation on β-actin gene expression may not unequivocally reflect what may be seen at the protein level. However, our observation may prompt efforts to investigate the effect of inflammation on protein expression to allow careful interpretation of Western blot results.

In conclusion, careful selection of the most suitable housekeeping genes is important in generating reliable results. The choice depends on the condition tested, the species and the organ under study.

Chapter 6

6. Effect of inflammation on molecular targets and drug transporters

6.1. Introduction

Inflammation is the host's response to infection, injury or irritation. The development of the inflammatory response is a complex process that involves increased expression of chemicals such as cytokines and chemokines in a paracrine, autocrine, and/or endocrine fashion. This enhances host's defense through immune cells recruitment and activation, antibodies production and complement system activation[17]. The process has been found to be associated with altered expression of other genes either as a part of the host's defense mechanism or because of shifting priorities in the cellular expression machinery. Examples of the affected genes are metabolizing enzymes[37, 96, 97], transporters[113], receptors[4, 5, 7, 9, 38] and plasma proteins[90, 91, 177]. Inflammation-induced alterations of gene expression may be responsible, at least in part, in disease pathogenesis. For example atrial fibrillation developed in septic shock patients[304, 305] has been attributed to inflammation-induced L-type calcium channel dysfunction[153]. In addition, animal and human studies have shown that inflammation is an important factor that may alter drug action and/or disposition in the body[3-6, 8, 52, 102, 191]. The purpose of the present work is to broaden the window of inflammation-drug interaction through characterizing the effect of inflammation on the molecular targets and transporters affecting the pharmacokinetics and pharmacodynamics of

drugs. We have used rats with pre-adjuvant arthritis (Pre-AA), an animal model of chronic inflammation that is known to mimic human rheumatoid arthritis[289].

6.2. Methods

6.2.1. Experimental Protocol

The study protocol was approved by the Health Sciences Animal Policy and Welfare Committee of the University of Alberta. Experiments were carried out on male Sprague-Dawley rats (220-280 g). They were housed in a controlled-temperature room with a 12-hours dark/light cycles. Two animal groups (n=4/group), inflamed (Pre-AA) and healthy (Control) were used. The inflamed group received 0.2 ml of 50 mg/ml *Mycobacterium butyricum* suspended in squalene into the tail base. Control animals received an equal volume of normal saline into the tail base. On day 12, rats were euthanized and their organs (hearts, liver, kidney and intestine) were excised and instantly frozen in liquid nitrogen. The excised organs were stored in -80 degrees.

6.2.2. Real time Polymerase Chain Reaction (PCR)

To determine the potential changes happening at the transcriptional level of the genes, RT-PCR was carried out. Total RNA was isolated from the frozen organs using TRIzol reagent (Invitrogen, Carlsband, CA, USA) according to the manufacturer's instructions (Chapter 3). This was followed by spectrophotometric quantitation of the isolated RNA by measuring the absorbance at 260 nm. cDNA was synthesized from 1.5 μg total RNA samples with the random primers scheme

using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Real Time PCR was performed on an ABI 7500 Real Time PCR system (Applied Biosystems, Foster City, CA, USA). Forward and reverse primers used for the experiments are depicted in Table 6-1. The housekeeping gene tested was the rat 18s rRNA. Melting curves were carried out to confirm amplification of single sequences and absence of primer dimers. Primers were purchased from Integrated DNA technologies (Coralville, IA, USA). PCR products were produced and detected quantitatively using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA). PCR data were analyzed using delta delta C_T method ($\Delta\Delta C_T$) as described by Livak and Schmittgen[247]. Experiments were done in duplicate for the genes showing a significant difference between the groups. The data are expressed as fold change relative to a calibrator (Control) and normalized to the housekeeping gene.

6.2.3. Data Analysis

Data are expressed as mean ± SD. Statistical significance between the control and inflamed groups was analyzed using a two-tailed student t-test. The homogenity of variance was tested using F-test. A *p* value of less than 0.05 was considered statistically significant. Satatistics were analyzed using Prism software v. 4.03 (GraphPad Software, Inc., San Diego, CA, USA).

Table 6-1. List of primers used in real-time PCR

Target	Forward Primer	Reverse Primer	Ref
$Ca_v1.2$	AGCAACTTCCCTCAGACGTTTG	GCTTCTCATGGGACGGTGAT	245, 306
β1-AR	CTGCTACAACGACCCCAAGTG	AACACCCGGAGGTACACGAA	307
β2-AR	GAGCCACACGGGAATGACA	CCAGGACGATAACCGACATGA	307
α_{1A}-AR	CGAATCCAGTGTCTTCGCAG	ACCATGTCTCTGTGCTGTCCC	308
$Na_v1.2$	TCCGGTTTCGTCACGCTATC	TCCAGAGAAGACTGATGTGACACC	309
$Na_v1.6$	CAAGCTGGAGAATGGAGGCA	TAAGAGGGGAGGGAGGCTGT	309
$K_v1.5$	CGCACAACGCTCGAGGAT	CCTCAGCAGATAGCCTTCTAGGTT	310
$K_v2.1$	ACGAGGGTCAGCTGCTCTACAG	TGCTGAACTTGGGACTGGTACTC	310
$K_v3.1$	CACGCATCTGGGCACTGTT	TGAAGAAGAGGGAGGCAAAGG	310
18s rRNA	GGGAGGTAGTGACGAAAAATAACAAT	TTGCCCTCCAATGGATCCT	290
oatp1a1	ACCTGGAACAGCAGTATGGAAAA	ACCGATAGGCAAAATGCTAGGTAT	311
oatp1a5	TGATGTGGATGGAACTAACA	TGCATTTATCTGGAGCACACTTG	311

	ATGAC		
oatp1b2	CCTGTTCAAGTTCATAGAGCA GCA	TGCCATAGTAGGTATGGTTATA ATTCCTAA	311, 312
oatp2b1	ACGACTTTGCCCACCATAGC	CCACGTAAAGGCGTAGCATGA	311
oatp4a1	AGAACGTCAAGTCGAGCTAT TCG	GGCCCACTTCTGTGTAAACATTT	311
oat2	CGTGTGTCCCAGGCATCA	TCCACACGACCCTGGGTTAG	311
oat3	GAGGACCTGTGATTGGAGAA CTG	CTGGCTGCCAGCATGAGATA	313
oct1	TGGTGTTCAGGCTGATGGAA	GCCCAAAACCCCAAACAAA	314
mdr1a	GTGGGAAAAGCACAACTGTC C	CCATGGTGACGTTTTCTCGG	311
bsep	CACTGGGTACATGTGGTGTCT CAT	ATGGCCAATATTCATAGCTGCTA AT	311
mrp1	CGAATGTCCTCTGAGATGGA GAC	CTCTACACGGCCTGAATGGG	311
mrp3	TCTTTGTGATGGCCTTGAGGA TA	TTGACCATTTCTCCCACAGTGT	311
mrp6	GAAGACTCCAAGCCATTGAA TCC	TCTCTCAAGTGACCAGAGGTCTT TT	311
IL-1α	AGGCATCCTCAGCAGCAGAA	AGCTCCACGGATGTGGAAAC	315
IFN-γ	GCCAAGTTCGAGGTGAACAA C	TAGATTCTGGTGACAGCTGGTG AA	316

iNOS	TGGTCCAACCTGCAGGTCTT	CAGTAATGGCCGACCTGATGT	317
MCP-1	CTGTCTCAGCCAGATGCAGTTAA	TGGGATCATCTTGCCAGTGA	316
IL-10	GAAGCTGAAGACCCTCTGGATACA	CCTTTGTCTTGGAGCTTATTAAAATCA	317
COX-1	TCCTGTTCCGAGCCCAGTT	CTTGGAAGGAATCAGGCATGA	318
COX-2	CCCCAAGGCACAAATATGATG	CCTCGCTTCTGATCTGTCTTGA	318
ACE	CACCGGCAAGGTCTGCTT	CTTGGCATAGTTTCG TGAGGAA	197
ACE-2	ACCCTTCTTACATCAGCCCTACTG	TGTCCAAAACCTACCCCACATAT	197

Primers were purchased from Integrated DNA Technologies (Coralville, IA, USA). Cav1.2, α1c subunit of L-type calcium channels; β1-AR, β1 adrenergic receptor, β2-AR, β2 adrenergic receptor; α1A-AR, α1A adrenergic receptor, Nav, voltage-gated sodium channels; Kv, voltage-gated potassium channels; 18s rRNA, 18s ribosomal RNA; oatp, organic anion transporter polypeptide; oat2, organic anion transporter; oct, organic cation transporter; mdr, multidrug resistance transporter; bsep, bile salts export pump; mrp, multidrug resistance-associated protein; IL, interleukin; IFN-γ, interferon γ; iNOS, inducible nitric oxide synthetase; MCP-1, monocyte chemoattractant protein 1; COX, cyclooxygenase; ACE, angiotensin converting enzyme.

6.3. Results

6.3.1. Constitutive Expression and Tissue Distribution of the Tested Genes in the Liver, Heart, Kidney and Intestine

Determination of the constitutive expression of the selected cytokines demonstrated basal levels of some of the examined genes in different organs (Figure 6-1). The constitutive expression of ACE, ACE-2, COX-1 and COX-2 (Figures 6-3a to 6-6a), drug transporters (Figure 6-7), adrenergic receptors (Figure 6-12) and voltage-gated ion channels (Figure 6-13) was found to be tissue specific. Generally, tissue distribution of drug transporters in the liver, kidney and intestine were found to be similar to those that have been previously reported (Table 6-2). In addition, we are reporting the presence of drug transporter genes such as oatp4a1, mdr1a, mrp1 and oatp2b1 in the heart.

Table 6-2. Tissue distribution and human orthologs of rat transporters.

Transporter	**Human ortholog**	**Constitutive gene distribution (in the present study)***	**Tissue distribution (reported elsewhere)**
Influx transporters:			
oatp1a1	OATP1B1, OATP1B3	Liver, kidney	Liver[319, 320], kidney[320], intestine[321], brain[322]
oatp1a5		Kidney	Intestine[321, 323], brain[321], lung[321]
oatp1b2	OATP1B1, OATP1B3	Liver, Kidney	Liver[129]
oatp2b1	OATP2B1	Liver, heart, kidney, intestine	Liver, intestine[324], placenta[325]
oatp4a1	OATP4A	Kidney, intestine, liver, heart	Ubiquitous[325]
oat2	OAT2	Liver, kidney, intestine	Liver[326], kidney[327]
oat3	OAT3	Kidney, liver	Liver[327], kidney[327], brain[328]
oct1	OCT1	Kidney, liver, intestine	Kidney[329], intestine[329]

Efflux transporters:

mdr1a	MDR1	Liver, intestine, kidney, heart	Ubiquitous[118, 330]
bsep	BSEP	Liver, kidney	Liver[331]
mrp1	MRP1	Kidney, heart, intestine, liver	Ubiquitous[319, 332]
mrp3	MRP3	Intestine, liver, kidney	Liver[319], kidney[333], intestine[334], brain[335]
mrp6	MRP6	Liver, intestine, kidney	Liver[319, 336]

*, mentioned in order of relative abundance. oatp, organic anion transporter polypeptide; oat2, organic anion transporter; oct, organic cation transporter; mdr, multidrug resistance transporter; bsep, bile salts export pump; mrp, multidrug resistance-associated protein.

6.3.2. Effect of Inflammation on the Tested Genes mRNA Expression in The Liver, Heart, Kidney and Intestine

Liver – As depicted in Figure 6-8, inflammation resulted in a significant reduction of oct1, oatp4a1 and mrp1 gene expression in the liver while mdr1a gene expression did not show any trend towards up- or downregulation. Generally, there was a trend towards drug transporter downregulation in the liver except for mrp3, mrp6 and mdr1a. However, the changes were not statistically significant. There was no difference between Pre-AA and control animals with respect to COX-1, COX-2, ion channels and adrenergic receptors gene expression (Figures 6-3b and 6-14a). ACE and ACE-2 gene expression were lower in inflamed animals but not to a significant degree (Figure 6-3b). However, ACE-2/ACE gene expression ratio in the liver of Pre-AA rats was significantly reduced from 0.7±0.1 (control) to 0.3±0.2 (Pre-AA) (Figure 6-15).

Kidney – As depicted in Figure 6-9, inflammation resulted in a significant reduction of oatp2b1, mrp6 and bsep gene expression in the kidney. On the other hand, oatp4a1 tended towards upregulation. That did not reach statistical significance. $Na_v1.2$ gene expression was significantly induced up to four fold in inflamed animals (Figure 6-14b). Generally, there was a trend towards renal cytokine gene upregulation in Pre-AA animals. However, only the elevation of IFN-γ was statistically significant (Figure 6-2b). There was no difference between Pre-AA and control animals with respect to COX-1, COX-2, other ion channels and adrenergic receptors gene expression (Figures 6-4b and 6-14b).

Heart – Out of the detected transporters in the hearts of control animals, oatp4a1 and mdr1a were found to be significantly increased by inflammation (Figure 6-10). mrp3 was not detected in inflamed animals. Interestingly, despite being undetected in control animals, oatp1b2, oat2 and oat3 were detected in inflamed rats. There was no significant difference between arthritis and control animals with respect to ion channels and adrenergic receptors gene expression (Figure 6-14c). While inflammation significantly inhibited COX-1 gene expression, it induced COX-2 genes (Figure 6-5b). Inflammation resulted in more than 80% reduction of ACE-2 gene expression in the rat heart (Figure 6-5b). There was an increased expression of ACE gene in Pre-AA animal hearts that did not reach statistical significance. However, ACE upregulation and ACE-2 downregulation resulted in a significant reduction in the ACE-2/ACE expression ratio from 0.7±0.4 in Control rats to 0.07±0.09 in Pre-AA rats (Figure 6-15).

Intestine – Owing to the high variability observed in intestinal tissue, none of the tested genes was significantly different from control. However, some transporters showed strong trend towards downregulation such as oct1, mdr1a and mrp3 (Figure 6-11).

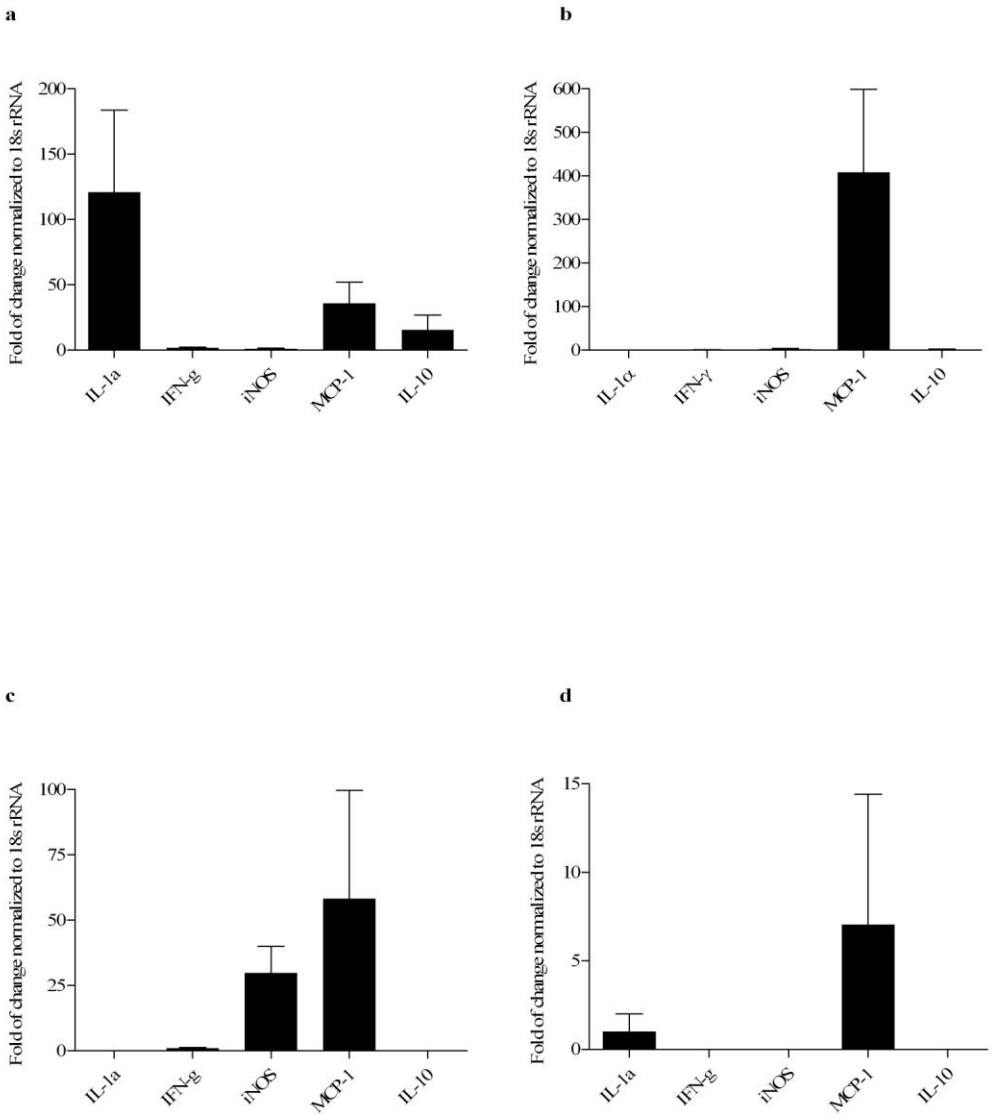


Figure 6-1. Constitutive expression of cytokine genes in different rat organs. Expression was determined using real time PCR in rat liver (a), kidney (b), heart (c) and intestine (d). Gene expression was normalized to 18s rRNA. Relative expression was determined relative to a calibrator, the gene with the lowest expression in each organ. (n=4/group)

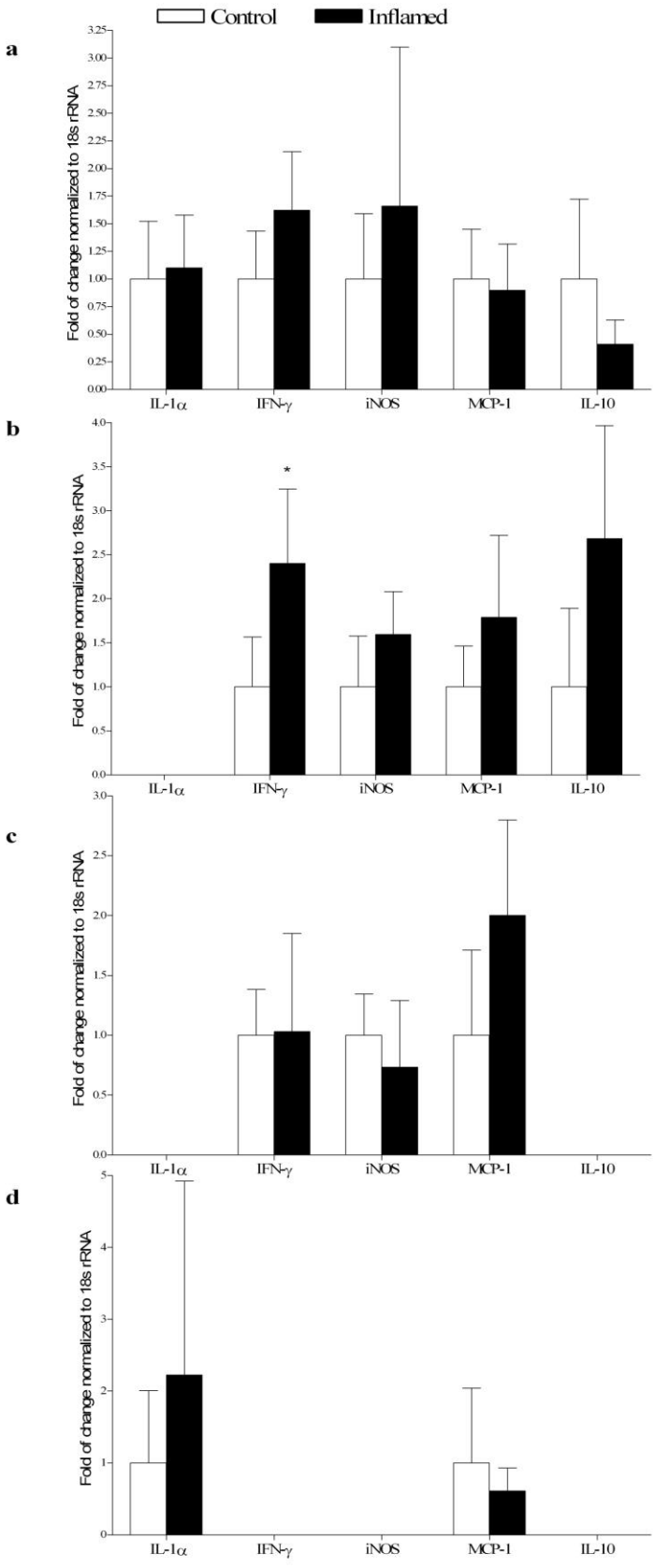
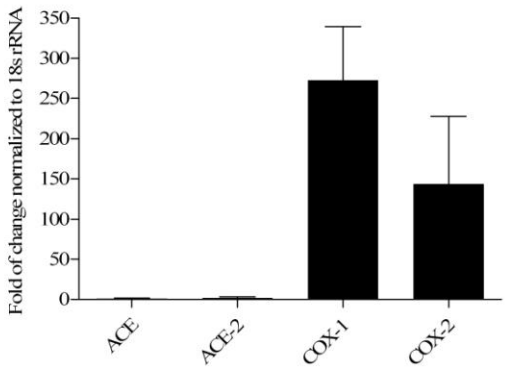

Control
Inflamed
a
b
c
d
Fold of change normalized to 18s rRNA
IL-1α
IFN-γ
iNOS
MCP-1
IL-10
*

Figure 6-2. Effect of pre-adjuvant arthritis on cytokine gene expression in different rat organs (n=4/group). Expression was determined using real time PCR in rat liver (a), kidney (b), heart (c) and intestine (d). Gene expression was normalized to 18s rRNA. *, $p<0.05$ vs. control rats.

a



b

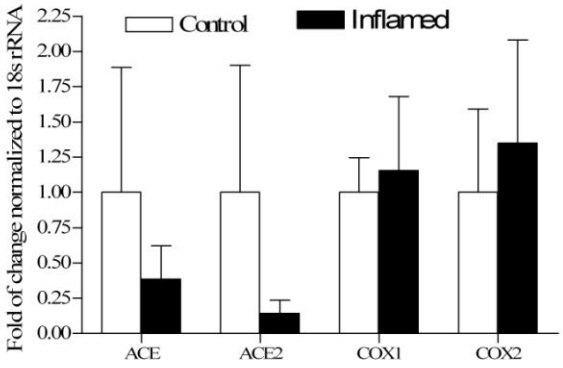
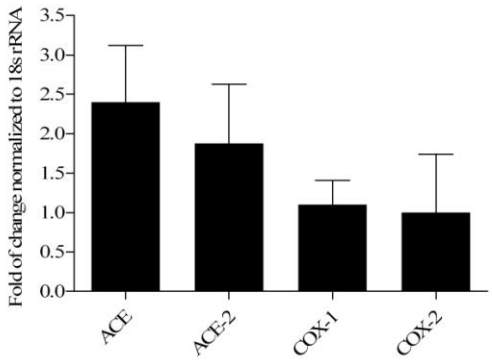


Figure 6-3. (a) Constitutive expression of angiotensin converting enzymes (ACE and ACE-2) and the cyclo-oxygenases (COX-1 and 2) genes in rat liver as determined by real time PCR. Relative expression was determined relative to a calibrator, the gene with the lowest expression. (b) Effect of pre-adjuvant arthritis on ACE, ACE-2, COX-1 and COX-2 gene expression in rat liver. Gene expression was normalized to 18s rRNA (n=4/group).

a



b

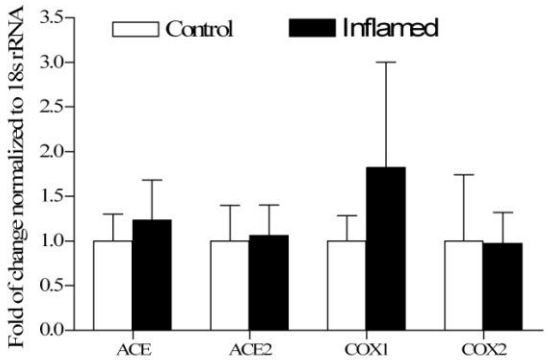


Figure 6-4. (a) Constitutive expression of angiotensin converting enzymes (ACE and ACE-2) and the cyclo-oxygenases (COX-1 and 2) genes in rat kidney as determined by real time PCR. Relative expression was determined relative to a calibrator, the gene with the lowest expression. (b) Effect of pre-adjuvant arthritis on ACE, ACE-2, COX-1 and COX-2 gene expression in rat kidney. Gene expression was normalized to 18s rRNA (n=4/group).

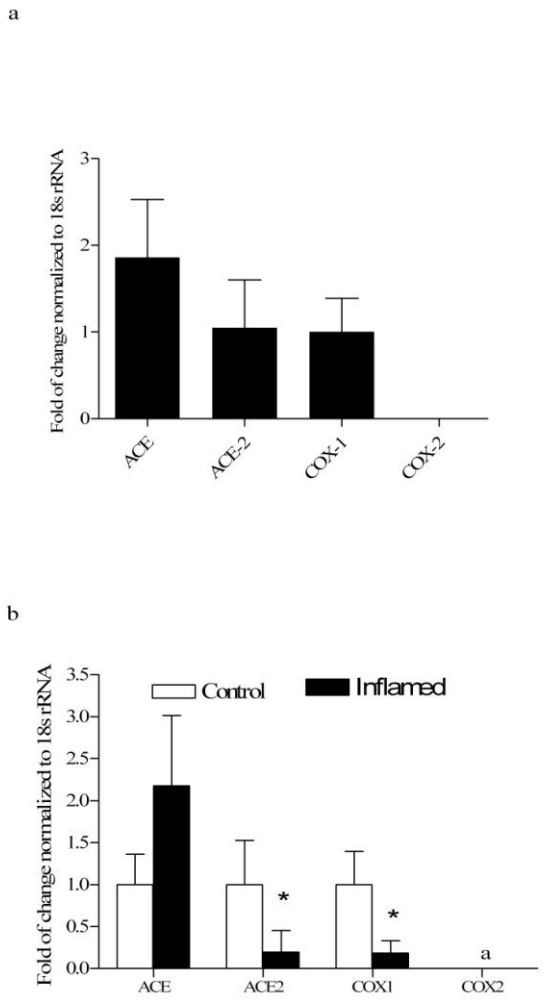
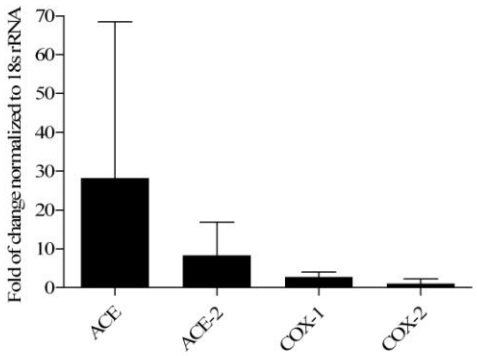


Figure 6-5. (a) Constitutive expression of angiotensin converting enzymes (ACE and ACE-2) and the cyclo-oxygenases (COX-1 and 2) genes in rat heart as determined by real time PCR. Relative expression was determined relative to a calibrator, the gene with the lowest expression. (b) Effect of pre-adjuvant arthritis on ACE, ACE-2, COX-1 and COX-2 gene expression in rat heart. Gene expression was normalized to 18s rRNA. *, $p<0.05$ vs. control rats. a, was detectable in pre-adjuvant arthritis group (n=4/group).

a



b

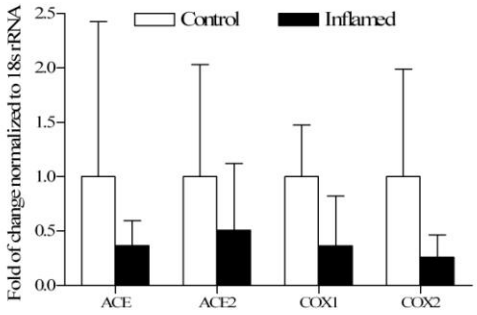


Figure 6-6. (a) Constitutive expression of angiotensin converting enzymes (ACE and ACE-2) and the cyclo-oxygenases (COX-1 and 2) genes in the rat intestine as determined by real time PCR (n=4/group). Relative expression was determined relative to a calibrator, the gene with the lowest expression. (b) Effect of pre-adjuvant arthritis on ACE, ACE-2, COX-1 and COX-2 gene expression in rat liver. Gene expression was normalized to 18s rRNA.

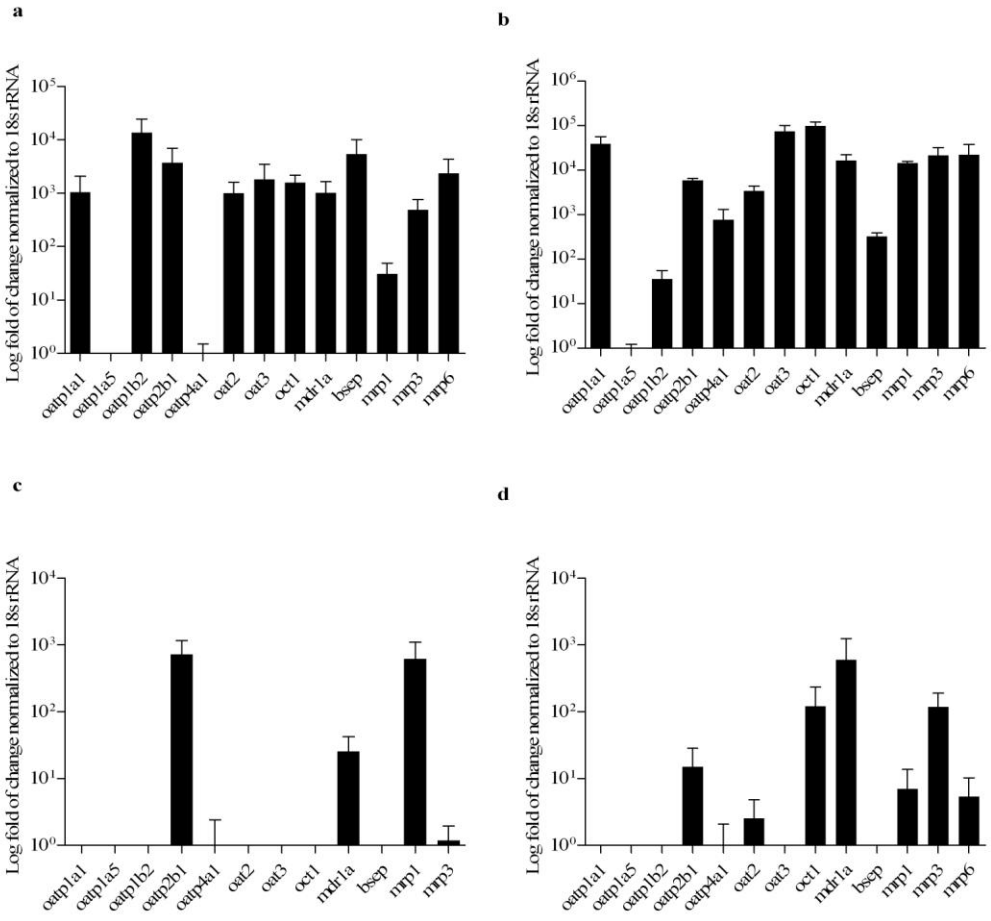


Figure 6-7. Constitutive expression of drug transporter genes in different rat organs (n=4/group). Expression was determined using real time PCR in rat liver (a), kidney (b), heart (c) and intestine (d). Gene expression was normalized to 18s rRNA. Relative expression was determined relative to a calibrator, the gene with the lowest expression in each organ.

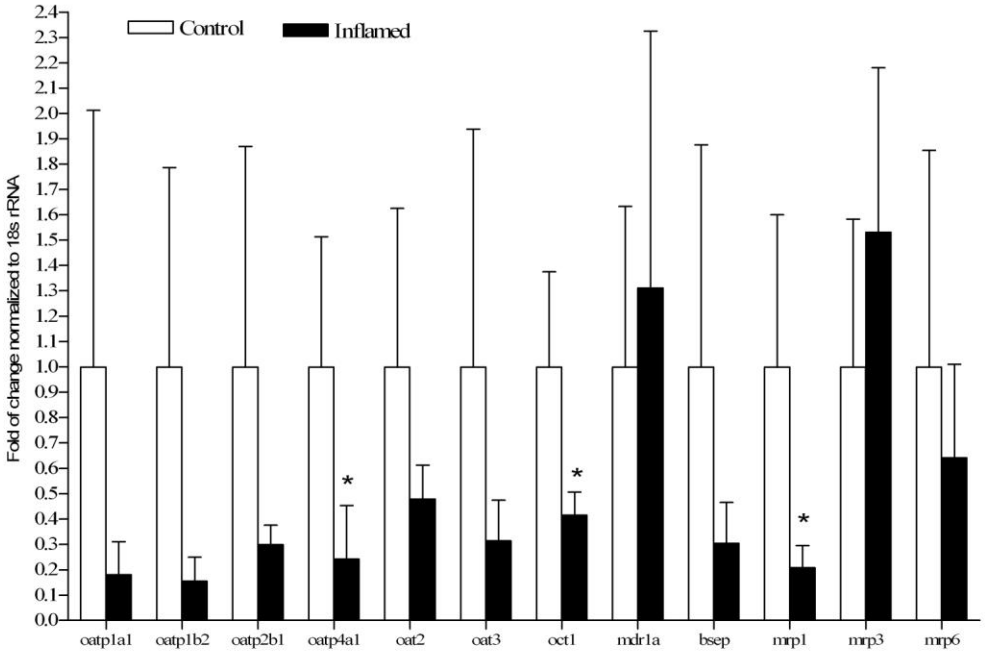


Figure 6-8. Effect of pre-adjuvant arthritis on drug transporter gene expression in rat liver as determined by real time PCR (n=4/group). Gene expression was normalized to 18s rRNA. *, $p<0.05$ vs. control rats.

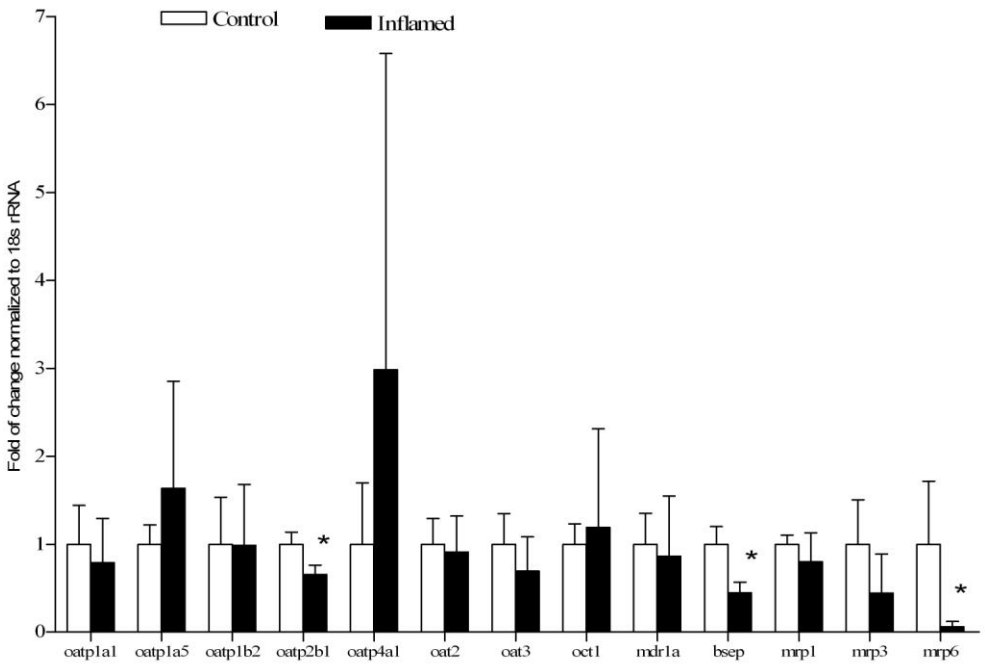


Figure 6-9. Effect of pre-adjuvant arthritis on drug transporter gene expression in rat kidney as determined by real time PCR (n=4/group). Gene expression was normalized to 18s rRNA. *, $p<0.05$ vs. control rats.

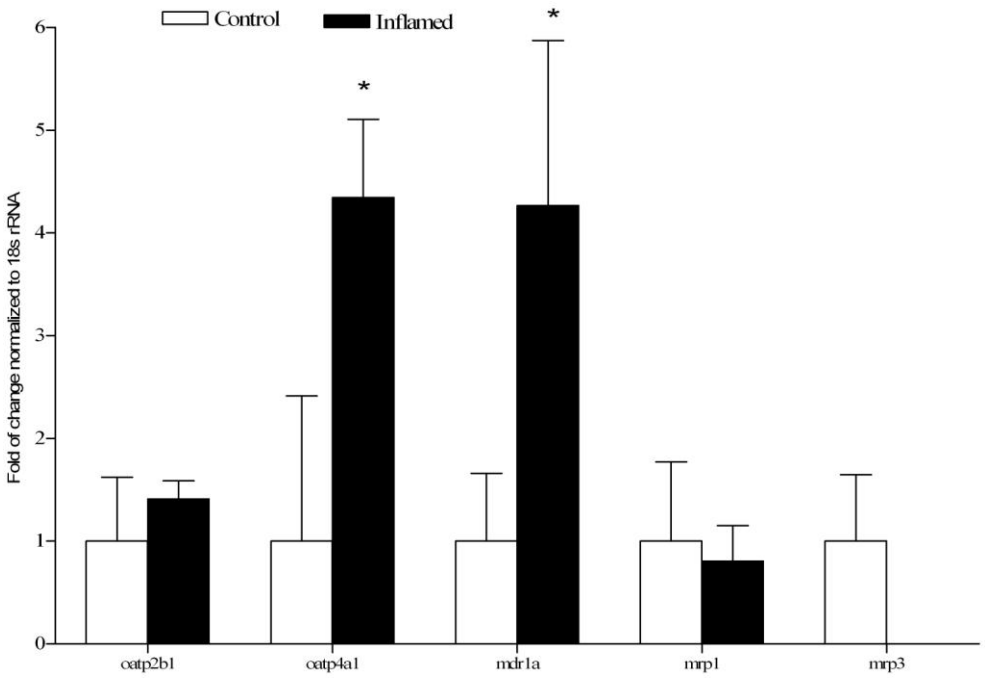


Figure 6-10. Effect of pre-adjuvant arthritis on drug transporter gene expression in rat heart as determined by real time PCR (n=4/group). Gene expression was normalized to 18s rRNA. *, $p<0.05$ vs. control rats.

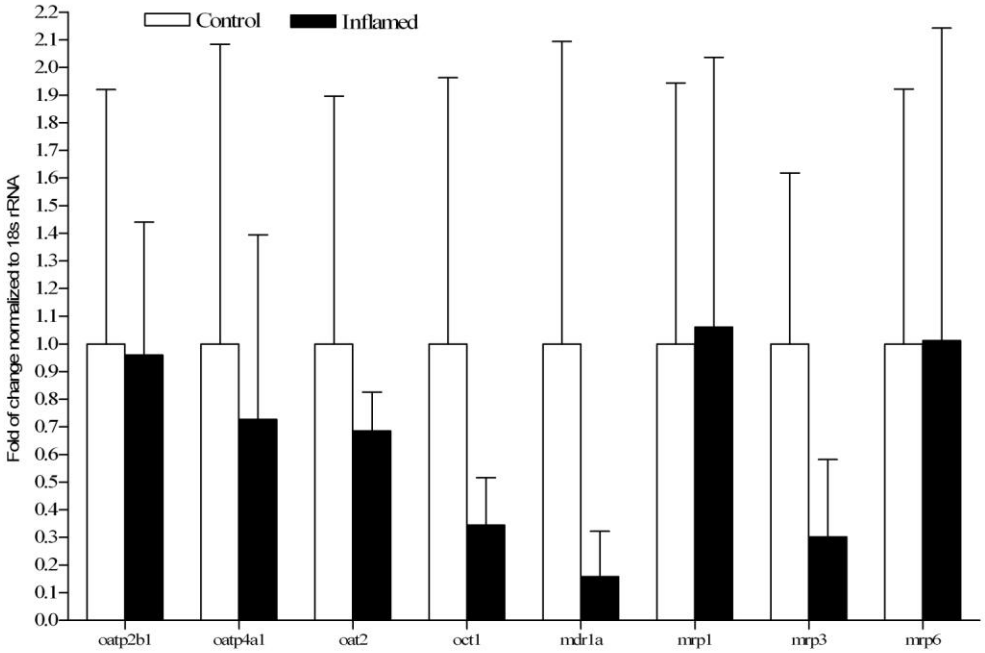


Figure 6-11. Effect of pre-adjuvant arthritis on drug transporter gene expression in rat intestine as determined by real time PCR (n=4/group). Gene expression was normalized to 18s rRNA.

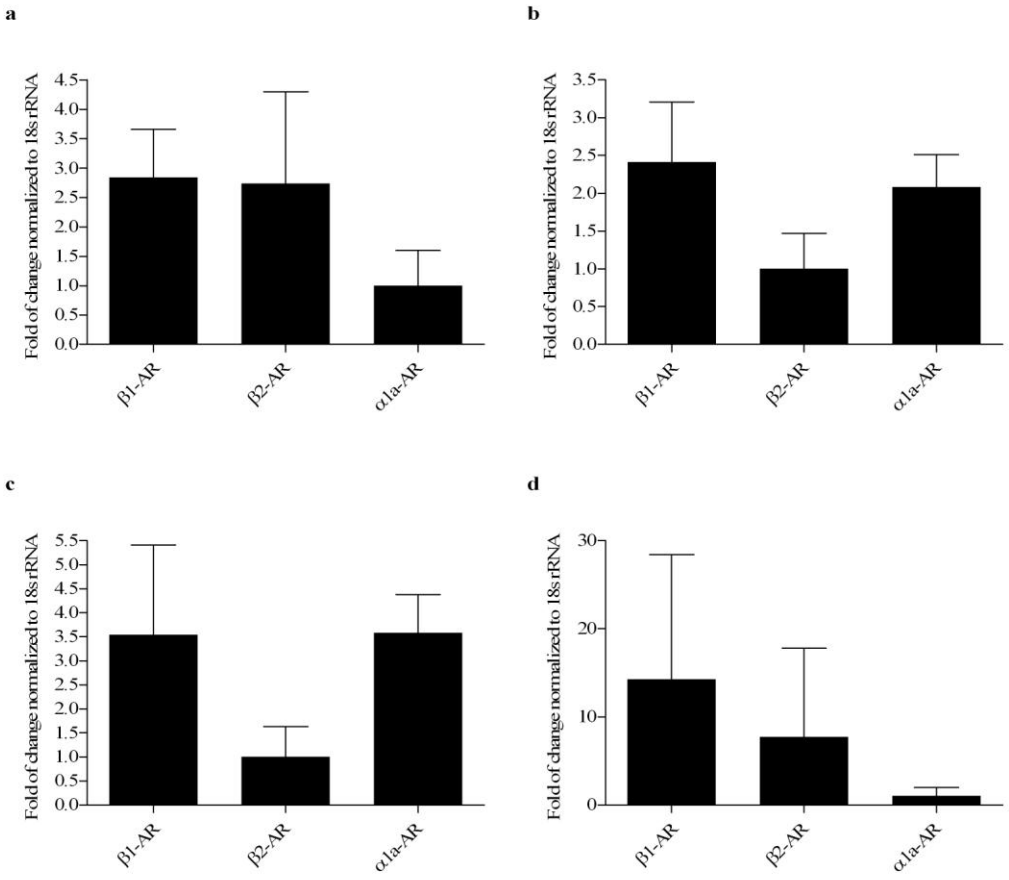


Figure 6-12. Constitutive expression of adrenergic receptors genes in different rat organs (n=4/group). Expression was determined using real time PCR in rat liver (a), kidney (b), heart (c) and intestine (d). Gene expression was normalized to 18s rRNA. Relative expression was determined relative to a calibrator, the gene with the lowest expression in each organ.

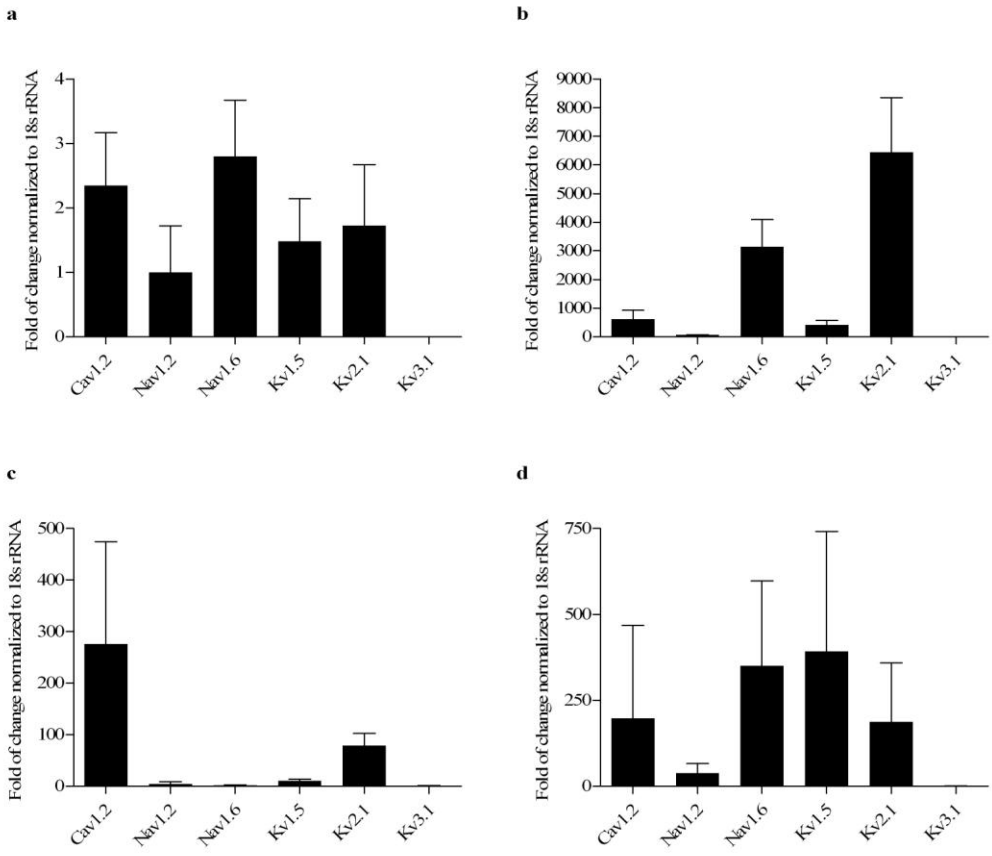


Figure 6-13. Constitutive expression of voltage gated ion channels genes in different rat organs (n=4/group). Expression was determined using real time PCR in rat liver (a), kidney (b), heart (c) and intestine (d). Gene expression was normalized to 18s rRNA. Relative expression was determined relative to a calibrator, the gene with the lowest expression in each organ (n=4/group).

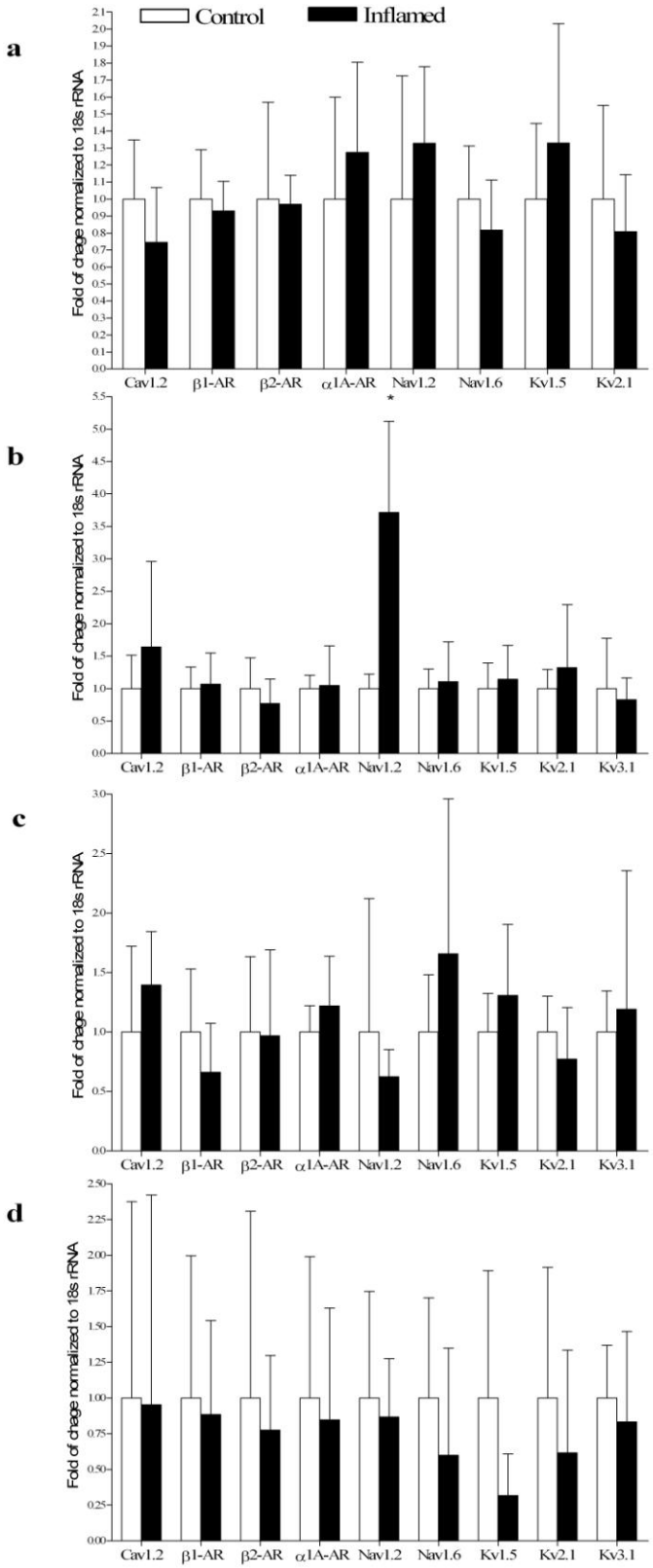

a
Control
Inflamed
Fold of chage normalized to 18s rRNA
Cav1.2
β1-AR
β2-AR
α1A-AR
Nav1.2
Nav1.6
Kv1.5
Kv2.1
b
*
Fold of chage normalized to 18s rRNA
Cav1.2
β1-AR
β2-AR
α1A-AR
Nav1.2
Nav1.6
Kv1.5
Kv2.1
Kv3.1
c
Fold of chage normalized to 18s rRNA
Cav1.2
β1-AR
β2-AR
α1A-AR
Nav1.2
Nav1.6
Kv1.5
Kv2.1
Kv3.1
d
Fold of chage normalized to 18s rRNA
Cav1.2
β1-AR
β2-AR
α1A-AR
Nav1.2
Nav1.6
Kv1.5
Kv2.1
Kv3.1

Figure 6-14. Effect of pre-adjuvant arthritis on adrenergic receptors and voltage gated ion channels gene expression in different rat organs (n=4/group). Expression was determined using real time PCR in rat liver (a), kidney (b), heart (c) and intestine (d). Gene expression was normalized to 18s rRNA. *, $p<0.05$ vs. control rats.

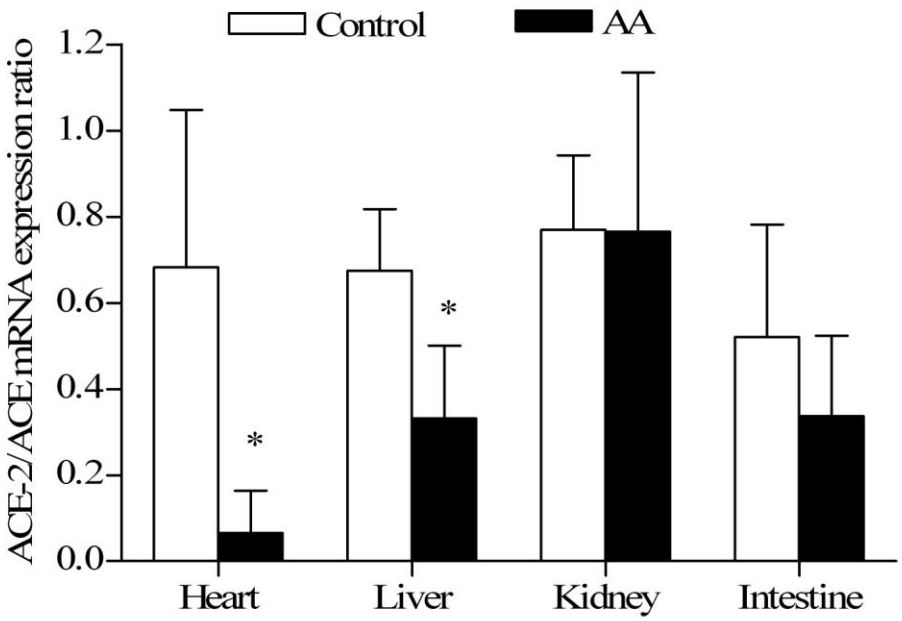


Figure 6-15. Effect of pre-adjuvant arthritis on ACE-2/ACE constitutive gene expression ratio in different rat organs (n=4/group). *, $p<0.05$ vs. control rats.

6.4. Discussion

Although a preliminary, this is an exploratory investigation into the general pattern of the effect of inflammatory conditions on the status of some important proteins. In this chapter, I have tried to explain the potential significance of each of the findings with full realization that my data are preliminary and further studies are required to confirm all the potential claims.

In the present study, we used Pre-AA, a model that mimics human rheumatoid arthritis in terms of development of systemic inflammation and arthritis[289]. Six days following administration of the pre-adjuvant, the inflammatory mediators start to rise[8] and after 12 days, animals develop chronic inflammation. This model allowed us to study the effect of chronic inflammation on the gene expression of drug transporters, adrenergic receptors, ion channels and select mediators of biological relevance. We studied the gene expression profile of iNOS and four cytokines. Our results showed that those genes are either not expressed in the studied organs or expressed in very little amounts (their C_T appeared in the last 10 cycles of the PCR run). However, MCP-1 was found to be constitutively expressed in the rat kidney, heart and intestine. This is in agreement to what has been previously reported in the rat interstitial fibroblasts[337] and smooth muscle cells[338] suggestive of a basal housekeeping function of the chemokine. Local expression of the inflammatory markers in the examined Pre-AA rat organs was not as pronounced as what has been previously reported in the

rat joints and lymphoid organs such as spleen and lymph nodes, the main sites of inflammation in pre-adjuvant arthritis[339]. However, few cytokine genes were found to be elevated in the tested organs of AA rats such as the significant increase of IFN-γ gene expression in the kidney of Pre-AA rats (Figure 6-2b). Similar changes were found for IFN-γ in the liver, MCP-1 in the kidney and heart and IL-1a in the intestine, but they did not reach statistical significance owing to experimental variability and differences in disease severities. It has been found that the serum concentrations of inflammatory markers such as TNF-α, IL-6 and serum nitrite are significantly elevated in pre-adjuvant arthritis rats[8, 9, 339]. This suggests that inflammation-induced alterations of target genes in those organs result mainly from the direct action of systemically elevated inflammatory mediators produced elsewhere rather their *de novo* synthesis in those organs.

6.4.1. Angiotensin Converting Enzymes (ACE and ACE-2)

The present work adds to the pathophysiology of inflammation another mechanism that may be responsible for inflammation-induced cardiovascular complications through inflammation-induced ACE-2/ACE imbalance. Renin angiotensin system (RAS) activation has been implicated in the pathogenesis of endothelial vascular dysfunction in inflammatory conditions[340]. Sakuta *et* al has found that the expression of the angiotensin converting enzyme (ACE) and the angiotensin II receptor 1 (AT1) is increased in the aorta of adjuvant arthritis rats contributing to endothelial dysfunction[340]. However, our finding that the expression of ACE-2 is also reduced added to the complexity of the RAS

system[196]. ACE and the newly discovered ACE-2[196, 197], are important enzymes involved in RAS, an important system involved in the regulation of blood pressure, electrolyte and fluid balance. ACE and ACE-2 catalyze different biological reactions[198]. ACE is a peptidyl dipeptidase that cleaves dipeptides from the C-terminal of angiotensin I converting it to the powerful vasoconstrictor angiotensin II (AGII). Moreover, it breaks down the vasodilator bradykinin adding to the vasoconstrictor response of AGII. On the other hand, ACE-2 converts angiotensin I to angiotensin (1-9) which is converted to angiotensin (1-7). The latter is also produced from the action of ACE-2 on AG II. Angiotensin (1-9) produced by ACE-2 has no known biological activity; however, angiotensin (1-7) is a vasodilator and has the reverse biological actions of AGII[198]. Crackower *et* al have examined ACE-2 knockout mice. They did not observe any change in blood pressure; however, knockout mice did have severe cardiac impairment suggesting the cardioprotective functions of ACE-2 [199]. On the other hand, the use of ACE and ACE-2 double knockout mice ablated the deleterious effects found in ACE-2 only knockouts. This suggests that ACE-2/ACE balance exists and is a part of maintaining the homeostasis in the cardiovascular system. Both ACE and ACE-2 are ubiquitously distributed in most tissues in human, rats and mice. However, tissue specific expression of these peptidases exists. ACE is most abundant in lungs, intestine, kidney, brain, aorta and adrenal medulla. ACE-2 is most abundant in intestine, lung, heart, kidney and placenta[341, 342].

We are reporting, for the first time, that inflammation results in more than 80% reduction of ACE-2 gene in the rat heart (Figure 6-5). Similar to what has been previously reported[340], ACE enzyme had a trend towards upregulation in the heart of Pre-AA rats (Figure 6-5). Although ACE changes were not significant, there was a significant increase in the gap between ACE and ACE-2 expression in the heart manifested by expression changes in opposite directions. ACE-2 has been found to provide negative feedback to the RAS and protection to the heart and kidneys [199, 343]. ACE-2 downregulation has been observed in other disease models such as experimental diabetic nephropathy and hypertension in rats[343]. Therefore, disruption of the balance between ACE-2/ACE observed in inflammation may be responsible, at least in part, for the cardiovascular complications observed in patients with inflammatory diseases. We have previously reported the beneficial effects of valsartan, an angiotensin II receptor blocker (ARB), in reversing the deleterious effects of inflammation in rats [9]. This can be explained, in light of the present report, that ARBs and ACE inhibitors can reverse ACE-2/ACE imbalance observed in inflammation.

Although we found a trend towards reduction of both ACE and ACE-2 in the livers and intestine of Pre-AA rats, ACE-2/ACE ratio was reduced substantially in rat livers. The pathophysiological consequences of this alteration are unknown. Despite being highly expressed in the intestinal tissues, ACE and ACE-2 physiological roles in the intestine are unknown. It has been reported that they help in peptide digestion because of their localization at the brush border of

the intestinal epithelia [344]. In addition, ACE-2 expression is increased in gastritis and inflammatory bowel diseases (IBDs) suggestive of the potential benefit of ACE-2 blockers in ameliorating IBDs [345].

6.4.2. Cyclooxygenases (COX-1 and COX-2)

Cyclooxygenases are important enzymes involved in the production of essential prostaglandins in all parts of the body. Cyclooxygenases exist in two forms, cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2). Cyclooxygenase-3 (COX-3) has been described recently as a splice variant of COX-1; however, its role is largely unknown. Generally, COX-1 is constitutively expressed in many tissues and COX-2 is the inducible form that is usually induced in different pathophysiological states such as inflammation and cancer[346]. In addition, COX-2 performs housekeeping functions and is constitutively expressed in the kidney and brain[347, 348]. In the present study, COX-1 gene was found to be constitutively expressed in the control rat liver, heart, kidney and intestine, while COX-2 was also expressed in all the examined tissues except the heart (Figures 6-3a to 6-6a). Interestingly, COX-2 genes were highly expressed in the kidney similar to COX-1 confirming the important function of COX-2 in rat macula densa as previously reported[348]. The observed tissue specific expression of COX-1 and COX-2 is also species specific. It has been reported that COX-1 gene is found in the liver, kidney and lung but not in the heart of Brown Norway and Fisher 344 rats and COX-2 has been found in the kidney, heart and lung but not in the liver in the same species[349]. COX-1 and COX-2 catalyze the conversion of arachidonic acid

to prostaglandin G_2 (PGG_2). The latter is then converted to the endoperoxide prostaglandin H_2 (PGH_2). Both PGG2 and PGH2 are highly unstable and can be easily converted by local enzymes to cell-specific prostanoids such as PGE2, thromboxanes and prostacyclin. Cellular specificity results from cell specific distribution of the terminal enzymes involved in prostaglandin biosynthesis[350]. It has been found that COX-2 is an important enzyme responsible for the development of inflammation in the Pre-AA model. COX-2 but not COX-1 expression is increased in Pre-AA rat paws and selective inhibition of COX-2 reverses the inflammatory response[339]. Determination of COX-1 and COX-2 genes expression in other AA rat organs has not been reported before. In the present study, neither COX-1 nor COX-2 gene expression was affected by inflammation in the liver (Figure 6-3b) and kidney (Figure 6-4b). In addition, in the rat intestine neither enzyme was significantly influenced by inflammation despite their trend towards downregulation. The abovementioned observations suggest that inflammation in the AA rats does not affect COX enzymes in the examined organs except for the heart. COX-1 enzyme in the rat heart was significantly downregulated by 80% and COX-2 expression was detected in AA rats. Similar observations have been reported in the hearts and lungs of rats treated with lipopolysaccharides[351, 352]. It has been found that COX-1 deficient mice have renal vasoconstriction and lower blood pressure reduction during sleep (non-dippers), a condition associated with increased risk of cardiovascular diseases in human[353]. The pathophysiological significance of the observed changes of COX enzymes in AA rat heart is unknown; however, the altered

balance may play a role in the pathogenesis of the cardiovascular complications observed in inflammatory diseases.

6.4.3. Drug Transporters[§]

6.4.3.1.Influx Transporters

Organic anion transporter polypeptides (OATPs) are a group of uptake transporters. They are responsible for the uptake of many endogenous and exogenous compounds (Table 6-3).

In the present study, oatp1a1 and oatp1b2 were found to be constitutively expressed in the rat liver and kidney in agreement to what has been previously reported[319, 320]. Rat oatp1a1 (previously known as oatp1), oatp1a4 (previously known as oatp2) and oatp1b2 (previously known as oatp4) are the rodent orthologs of human OATP1B1 and OATP1B3[355, 356]. Their clinical importance has been described in genetic polymorphism. For example, polymorphism in OATP1B3 is associated with an increase in pravastatin plasma level[357]. Oatp1a1 has been found to be downregulated in extrahepatic cholestasis, an effect mediated by TNF-α[358]. Nevertheless, downregulation of a certain transporter does not mean alteration of clearance and pharmacokinetics of its substrates. For example, downregulation of oatp1a1 does not affect the capacity of bile salt transport because bile salts hepatic influx is also mediated by other transporters that are not affected by inflammation[358]. Oatp1b2 has been found to be

[§] Human and rodents transporters are represented in the text by upper case and lower case letters, respectively.

responsible for the hepatic uptake of pravastatin[359]. The use of oatp1b2 knockout mice resulted in 4 and 8-fold decrease of the liver to plasma ratio of pravastatin and rifampicin, respectively and a 43% reduction of rifampicin clearance[360]. Moreover, knockout animals are less sensitive to the hepatotoxic effects of some toxins due to their decreased uptake[359]. Hepatic oatp1a1 and 1b2 were found to undergo a trend towards downregulation in Pre-AA rats; however, due to experimental variability the difference was not statistically significant. On the other hands, neither transporter expression was altered in the kidney, suggestive of an unaltered transport of their substrates in chronic inflammation. In contrast to oatp1a1 and 1b2, oatp2b1 was found to be constitutively expressed in all the examined rat organs (Figure 6-7). It has been previously reported that it is expressed in the rat liver, intestine and placenta[321, 324]. Oatp2b1 is an important transporter involved in the cellular uptake of digoxin[324]. To the best of our knowledge, we are reporting, for the first time, the expression of oatp2b1 in the rat heart. Cardiac oatp2b1 expression may imply its role in the cardiac uptake of digoxin. The expression of oatp2b1 was significantly reduced in the kidney of Pre-AA rats, an observation that may explain, at least in part, the previously reported reduced digoxin clearance in TNF-α treated mice[361]. Oatp1a5 (previously known as oatp3) has been found to be involved in the intestinal absorption of fexofenadine in rats[362]. It was not detected in the tested organs and showed a very little expression in the kidney. However, it has been reported that it is expressed in the lung, brain and intestine[321, 323]. The reason for the discrepancy in the

intestine can be explained by its differential expression in different areas in the rat intestine.

OATP4A1 is one of the thyroid hormone transporters[363]. Thyroid hormones T3 and T4 are substrates of several uptake transporters including L-amino acid transporters (LAT1, LAT2), monocarboxylate transporters (MCT8) and several OATPs[363]. Thyroid hormones are transported in the rat by oatp1a1, oatp1a4, oatp1a5, oatp1b2, oatp1c1, oatp4a1, oatp4c1, oatp6b1, oatp6c1, lat1, lat2 and mct8[363]. Euthyroid sick syndrome is a condition characterized by altered thyroid hormone levels with normal thyroid gland function. It has been found in patients with various inflammatory conditions such as acute myocardial infarction, trauma and infection[364]. Inflammation has been implicated in the development of euthyroid sick syndrome. For example, lipopolysaccharides administration inhibited the hypothalamic pituitary- thyroid axis causing decreased TSH, T3 and T4[365, 366]. In addition, rheumatoid arthritis is associated with thyroid gland abnormalities[367-369]. This has been explained, in part, to be due to an inflammation-induced upregulation of thyroid hormone transporters leading to increased cellular uptake of thyroid hormones. For example, MCT8 in the liver and skeletal muscles has been found to be significantly upregulated in patients with critical illness[370]. These observations are in agreement with what we have found in the Pre-AA rat hearts and the upregulation trend in the kidney. On the other hand, oap4a1 was significantly downregulated in the liver of Pre-AA rats, a

process if combined with changes to other thyroid hormone transporters, may significantly alter the hepatic uptake of thyroid hormones.

Organic anion transporters (OATs) and organic cation transporters (OCTs) are important uptake transporters involved in the carrier-mediated transport of many endogenous and exogenous compounds (Table 6-3). In the present study, we determined the expression profile of three key transporters, oat2, oat3 and oct1. In agreement to what has been previously reported[326, 327, 329], oat2, oat3 and oct1 were found to be expressed in the healthy rat liver and kidney. Oat2 and oat3 are expressed in the basolateral membrane of renal tubular cells and are responsible for the uptake of organic anions from the blood stream for subsequent excretion in urine[371]. Downregulation of OATs or their inhibition has been found to result in a reduction of the renal clearance of their substrates. For example probencid administration, an OAT inhibitor, inhibits the renal excretion of penicillin G and ACE inhibitors and prolongs their half-lives[372]. The renal uptake of xenobiotics mediated by OATs has also been described as a means for nephrotoxins accumulation in the kidney and contribution to kidney failure[373]. In the liver, ion transporters are responsible for the sinusoidal uptake of drugs and endogenous compounds[371]. Moreover, oat2 and oct1 were found to be expressed in the rat intestine (Figure 6-7d) suggesting their role in facilitating drug absorption. In the intestine, neither transporter was affected by inflammation in Pre-AA rats. Similarly, OCT1 is not downregulated in the intestinal tissue of inflammatory bowel disease patienats[114]. The lack of inflammation-induced

alterations of the renal oat and oct indicates that the renal excretion of their drug substrates is not affected in Pre-AA rats. This can explain, at least in part, the unaltered renal clearance of renally excreted drugs in inflammatory conditions such as sotalol, valsartan and losartan[5, 221, 222]. In contrast to the kidney, oct1 is significantly downregulated in the liver of Pre-AA rats suggesting the potential reduction of the hepatic uptake of its substrates (Table 6-3). TNF-α and IL-6 have been implicated in inflammation-induced downregulation of hepatic OATs and OCTs in humans. Incubation of human hepatocytes with TNF-α or IL-6 resulted in reduced expression of OCT1 and OAT2[374]. On the other hand, lipopolysaccharides administration in rats did not alter oat2 mRNA[375] suggesting the dependency of the observed effects on the model examined and the cytokine release profile.

6.4.3.2.Efflux Transporters

In the present study, we examined the effect of inflammation on the expression of five efflux transporters, mdr1a, bsep, mrp1, mrp3 and mrp6. Mdr1a and mdr1b are the rodent orthologs of human MDR1 (p-glycoprotein, P-gp). However, mdr1a is the predominant transporter in Sprague Dawley rats[376]. P-gp is the most studied efflux transporter owing to its ubiquitous distribution in most body tissues in humans and animals, its broad substrate specificity and its association with numerous clinically relevant drug interactions. Mdr1a is strategically located in different organs to facilitate specific functions. It is localized in the apical side of hepatocytes, renal tubular and intestinal cells and is responsible for the biliary

secretion, tubular secretion and resisting absorption of drugs and their metabolites, respectively. The clinical significance of P-gp has been investigated by the use of P-gp inhibitors and P-gp knockout animals. For example, injection of radiolabeled digoxin and cyclosporine in mdr1a knockout animals resulted in 20 to 50 fold increase of drug exposure[377]. The effect of inflammation on P-gp expression has been found to be model, species and organ dependent[5, 113]. For example, acute inflammatory models in rats (turpentine-induced[115] and endotoxin-induced[54, 114-116]) have been associated with reduced expression and activity of P-gp in the rat liver, upper GI tract and kidney. On the other hand, endotoxin-induced inflammation in mice has been associated with upregulation of renal mdr1a and increased doxorubicin renal clearance[120]. Other rodent models such as renal failure[121], colitis[122] have been found to be associated with reduced expression of P-gp in intestinal tissues. However, no change has been found in experimental cholestasis[119]. The reason for these discrepancies has been explained, in part, by the variation in inflammatory mediator profiles in different diseases and models of inflammation[378]. For example, while in vitro incubation of IL-6 with hepatocytes resulted in mdr1a downregulation[287, 379], IFN-γ incubation resulted in increased mRNA and protein expression with no change in the activity in Caco-2 cells[125]. The effect of chronic inflammation on P-gp gene expression in Pre-AA rat model has not been studied before. In the present study, mdra1 gene expression was not altered in the rat liver and kidney, the two major elimination organs suggestive of unaltered biliary clearance and tubular secretion of mdr1a substrates. Interestingly, P-gp mRNA was significantly increased by more than 4-

fold in the hearts of Pre-AA rats in contrast to what has been previously reported in a rat model of acute inflammation[380]. This may, at least in part, explain the observed reduced response of cardiovascular drugs in Pre-AA rats[3-6, 9, 52]. However, inflammation-induced pharmacodynamic alterations, target channel or receptor downregulation, have higher impact than the reduced cardiac uptake. For example, the reduced verapamil response in rats with endotoxemia has been found to be independent of its reduced uptake[381]. On the other hand, altered cardiac P-gp function can have a crucial role in determining drug cardiotoxicities as in the case of idarubicin[382].

BSEP, formerly known as the sister of P-glycoprotein, is an important efflux transporter located at the canalicular side of hepatocytes and is involved mainly in the hepatic secretion of bile salts[383]. In addition to bile salts transport, it is involved in pravastatin[384] and vinblastine[385] transport. Generally, BSEP is exclusively expressed in the liver. However, tissue distribution studies have detected its presence in the rat gut[386]. Interestingly, in addition to the liver, we report for the first time the extrahepatic expression of BSEP the rat kidney (Figure 6-7b). However, the biological function of bsep in the kidney and intestine is not known. Lipopolysaccharides administration in rats resulted in a decline in bsep expression, an effect that can explain LPS-induced intrahepatic cholestasis[375]. In the present study, BSEP is downregulated in the liver and kidney of Pre-AA rats. This alteration may affect bile salt transport in Pre-AA rat livers and may lead to cholestasis similar to those produced in rats by estrogen and troglitazone, bsep

inhibitors[387, 388]. The consequences of the reduced function of BSEP are species dependent. While BSEP gene mutation or inhibition in human can lead to progressive familial intrahepatic cholestasis or drug-induced cholestasis, respectively, bsep knockout mice experienced only mild cholestasis[389, 390]. The latter has been explained by the compensatory effects of p-glycoprotein in bile salt transport in mice[389].

Multidrug resistance-associated proteins (MRPs) are a group of efflux transporters of the ATP-binding cassette superfamily. Their biological function depends on their cellular localization. To date, there are nine discovered MRPs (MRP1 to MRP9) in human. MRPs are widely distributed in the body with differential expression in many organs such as the liver, kidney, brain and intestine. They are strategically localized in the basolateral and/or apical sides of the hepatocytes, renal tubular cells, intestinal epithelial cells, blood brain barrier epithelial cells to perform different biological functions[391-394]. Similarly, we found that mrp1, mrp3 and mrp6 are constitutively expressed in the rat liver, kidney and intestine (Figure 6-7). Mrp1 and mrp3 are also expressed in the heart of control rats. MRP6 is located in the lateral and canlicular sides of hepatocytes in rat liver. It has been postulated that MRP6 serves a housekeeping function in solute transport into bile[336]. MRPs have been implicated in human diseases. For example, MRP2 plays a role in the pathogenesis of Dubin-Johnson syndrome, a condition characterized by conjugated hyperbilirubinemia and MRP6 dysfunction has been implicated in the development of pseudoxanthoma elasticum, a genetic

condition characterized by elastic fiber fragmentation and premature atherosclerosis[112]. In addition, MRP1 has been associated with the resistance of tumor cells to anticancer chemotherapy by reducing their cellular accumulation[112]. Several anticancer agents are MRP1 substrates such as vincristine, etoposide, doxorubicin and daunorubicin. mrp1 gene expression was significantly reduced by more than 75% in the liver of Pre-AA rats and mrp3 had a trend towards upregulation but it did not reach statistical significance (Figure 6-8). Both MRP1 and MRP3 are localized on the basolateral side of hepatocytes and so they are responsible for the efflux of chemicals to the blood stream[319]. Therefore, inflammation-induced up- or downregulation of those transporters may alter the hepatic handling of their substrates. It has been reported that inflammation can alter the expression of MRPs in vitro and in vivo. However, the results obtained from other studies were controversial. It has been found that human hepatoma cells treated with IL-6 is associated with upregulation of both MRP1 and MRP3 in vitro[128]. Also, upregulated mrp1 mRNA has been detected in the endotoxemic rat liver[127]. This confirms the notion that studying the effect of inflammation on gene expression is a model and disease dependent. The role of MRP1 in inflammatory diseases is rather complex. It has been found that MRP1 plays a role in the pathogenesis of inflammation. Glutathione and its conjugates are transported by MRP1, as a part in the detoxification pathway of toxins[395]. The role of MRP1 in inflammation is model and disease dependent. While mrp1 knockout mice exhibited blunted response to arachidonic acid-induced inflammation[396], they manifested aggravated intestinal inflammation to dextran

sulfate[397]. Therefore, inflammation-induced alterations of MRPs expression may be a part of the host defense against injurious agents.

Table 6-3 Substrates of the tested drug transporters[354].

Transporter	Drug substrates
oatp1a1	Enalapril, indomethacin, PG E2 methotrexate, dexamethasone, cortisol, aldosterone, digoxin, T3, T4, Bile salts, pravastatin
oatp1a5	Bile salts, methotrexate, digoxin, T3, T4
oatp1b2	T3, T4, digoxin, methotrexate
oatp2b1	PG F2α, PG E2, digoxin
oatp4a1	T3, taurocholate
oat2	PG F2α, salicylate, PG E2, methotrexate, acetyl salicylate, cimetidine, indomethacin
oat3	Pravastatin, penicillin G, cimetidine
oct1	Cytarabine, metformin, amantadine, quinine, quinidine, dopamine, pnacuronium, cimetidine, saquinavir, pramipexole
mdr1a	Quinidine, verapamil, octreotide
bsep	Pravastatin, bile salts
mrp1	Vincristine, etoposide, doxorubicin, daunorubicin, 17β-estradiole conjugate
mrp3	17β estradiole conjugate, methotrexate
mrp6	Anthracyclines

PG, prostaglandin; oatp, organic anion transporter polypeptide; oat2, organic anion transporter; oct, organic cation transporter; mdr, multidrug resistance transporter; bsep, bile salts export pump; mrp, multidrug resistance-associated protein.

6.4.4. Ion Channels

L-type calcium channels are widely distributed in the body but mainly localized in cardiac muscles, endocrine cells and neurons[129, 130]. L-type calcium channels are essential for coupling the membrane excitation to muscle contraction especially in cardiac muscles. In addition, calcium entry through those channels is responsible for neurotransmitter release, synaptic plasticity and gene expression. Increased influx of calcium is also responsible for the phase 2 plateau in myocytes action potential and AV-nodal conduction[134, 136]. We examined the gene expression of the alpha subunit of L-type calcium channels ($Ca_v1.2$), the functioning subunit of the channel. RT-PCR detected $Ca_v1.2$ gene in the rat heart, kidney, liver and intestine as previously reported[136, 398]. There was no significant change in mRNA of $Ca_v1.2$ following induction of inflammation. However, our previous studies showed that $Ca_v1.2$ protein is downregulated in Pre-AA[9] and the acute myocardial injury rats (Chapter 3). In addition, other inflammatory models have shown reduced L-type calcium channel currents in ventricular myocytes by endotoxins and in patients with atrial fibrillation and severe heart disease[132, 133]. In agreement to our present observation, in post-AMI rats, despite its protein downregulation, $Ca_v1.2$ mRNA was not significantly different suggesting that inflammation induced alterations are at the post-transcriptional (translational) level (Chapter 3). Moreover, previous reports have shown that mRNA of $Ca_v1.2$ can rapidly change[153, 235]. For example, 6 hrs following lipopolysaccharides injection, $Ca_v1.2$ mRNA were downregulated then returned back to normal in 24 hrs[153]. Besides direct effects on the channels, inflammation can alter the downstream signaling

mechanisms affecting calcium channel functions. Acetylcholine exposure augments calcium current in normal but not inflamed dogs. This has been explained by the alteration of the second messenger system coupling muscarinic receptor activation to L-type calcium channels[151]. Therefore, the above finding combined with our studies in Chapters 2 and 3, added to our understanding of the possible mechanisms of reduced calcium channel function.

In addition to calcium channels, we examined the gene expression of two subtypes of voltage-gated sodium channels, $Na_v1.2$ and $Na_v1.6$ and three subtypes of voltage-gated potassium channels, $K_v1.5$, $K_v2.1$ and $K_v3.1$. It has been previously reported that $Na_v1.2$ and $Na_v1.6$ are expressed in the rat brain[399]. They function in the action potential initiation and conduction. Both channels are actual and potential targets for antiepileptic drugs. We report for the first time the expression of both channels in the rat liver and to a lesser extent in the kidney, heart and intestine. Using RT-PCR we did not find any changes in Pre-AA rats except that $Na_v1.2$ is significantly upregulated in Pre-AA rat kidneys (Figure 6-14b). The unaltered sodium channel expression in inflamed animals, suggests unaltered pharmacological effects of drugs acting on those receptors. For example, the pharmacological effects of lidocaine, a voltage-gated sodium channel blocker, are not affected by inflammation[400]. While, Kv1.5 is distributed in the kidney, gastrointestinal tract, heart, smooth muscles, CNS and blood vessels[401, 402], $K_v2.1$ is distributed in the brain, heart, pulmonary arterial smooth muscles and skeletal muscles[401]. $Ka_v1.5$, an ultra rapid delayed rectifier channel,

plays a role in maintaining the membrane potential and excitability of neurons and cardiac muscles[403]. It is considered a potential target for treatment of atrial fibrillation. Potassium channels alterations have been implicated in human diseases. While, $K_v1.5$ is downregulated in patients with atrial fibrillation[140, 141], both $K_v1.5$ and $K_v2.1$ are downregulated in chronic pulmonary hypertension[114, 115]. There was no significant difference between control and inflamed animals among the examined potassium channels. Absence of change in expression may not be extrapolated at the protein level. For example, Brundel et al has found a discrepancy in $Ka_v1.5$ gene and protein expression in the hearts of patients with atrial fibrillation. While, $K_v1.5$ mRNA did not change, its protein is downregulated[404].

6.4.5. Adrenergic Receptors

In the present study, we tested the gene expression profile of three adrenergic receptors, β1, β2 and α_{1A} receptors. Each receptor provides important physiological functions mediated by the sympathetic nervous system. They are considered the main site of action of many drugs that are clinically in use such as β1 adrenoceptor and α1-blockers. Similar to L-type calcium channels, inflammation did not affect the mRNA expression of adrenergic receptors. However, it has been found that propranolol potency, a β1 adrenoceptor blocker, is reduced in inflammation[6, 52] and this has been attributed to reduced expression of β1 receptor proteins[405]. The reason for this discrepancy can be explained at the

level of protein rather than the gene, in other words, the effect is post transcriptional as in the case of L-type calcium channels.

In conclusion, inflammation, the host defense mechanism against exogenous pathogens, alters the gene expression of many mediators, drug transporters and ion channels that can influence the behavior of drugs in the body and contribute to therapeutic failure.

Chapter 7

7. General Conclusions

Inflammation is the physiological reaction of the body towards external and internal stimuli. It is the normal defense mechanism that the body uses to defend it from infections and foreign bodies. However, the inflammatory response may become exaggerated causing harm to the body. Inflammation is a broad phenomenon that complicates a tremendous array of conditions and diseases ranging from obesity to cancer. Inflammatory conditions are all associated with the release of cytokines and other inflammatory markers that play important roles in the pathogenesis and progression of those conditions. In addition to their function in immuno-modulation and immunity-related diseases, inflammatory mediators can alter the phenotype, function and/or gene expression of non-immune cells leading to a huge array of effects and adverse effects. The differences in the activated inflammatory pathways, cytokine release profile, Th1/Th2 balance shift and the severity of the injury shape the clinical presentation of the condition. Each inflammatory reaction is, therefore, unique and can alter cellular protein expression in a different way. Of those altered proteins are drug targets, receptors, metabolizing enzymes and transporters leading to altered drug action and disposition in the body. Disease-drug interaction is an important challenge that faces the success of pharmacotherapy in patients with inflammatory diseases. Since patients with inflammatory conditions can have superimposing diseases that need drug therapy, the study of the effect of inflammation on drugs pharmacokinetics and pharmacodynamics becomes pivotal. For example

rheumatoid arthritis patients are at increased risk of cardiovascular diseases and may require the use of calcium channel blockers where their potency is compromised in inflammation.

In the present research, we have examined arthritis, myocardial injury and pediatric obesity, three conditions that have one feature in common, inflammation. In the animal arm of the study, the pharmacological potency of verapamil is reduced in pre-adjuvant arthritis rats, an effect similar to what have been previously reported in other animal models of arthritis and in human active rheumatoid arthritis. In addition, verapamil potency is compromised in post acute myocardial injury rats. This observation may not unequivocally reflect what may be seen in humans. However, since the disease is associated with inflammation and the reduced response is noted in various types of humans and animals inflammatory conditions, our observation may prompt efforts to investigate reduced drug response as a potential contributory factor in poor therapeutic outcome in post myocardial infarction patients. Similarly, in the clinical arm of the study, the antihypertensive potency of calcium channel blockers is also reduced. Therefore, obesity may be considered when initiating antihypertensive drug therapy for children especially calcium channel blockers such as nifedipine and amlodipine are the most commonly prescribed drugs to treat hypertension in children. The above findings confirm the notion that inflammation-drug interaction is not exclusive to a specific inflammatory condition. In the present research, we were focused on cardiovascular drugs notably calcium channel

blockers. We found that the altered response to calcium channel blockers can be explained at the pharmacodynamic level where the target protein, L-type calcium channels, is downregulated in Pre-AA and AMI rats.

The aim of pharmacotherapy of inflammatory conditions is to control the inflammation, thereby, stop the progression of the disease. This is also expected to restore the altered drug actions and disposition. In the present study, we tested the hypothesis that controlling inflammation using valsartan treatment can restore the previously reported altered verapamil pharmacokinetics and pharmacodynamics. Such an effect is expected due to the anti-inflammatory properties of angiotensin II inhibition. A six-day valsartan treatment reversed the observed downregulation of L-type calcium channels in Pre-AA rats, a finding that explained the observed enhanced verapamil potency in valsartan-treated animals. This beneficial interaction of valsartan with calcium channels blockers, once also proven in humans, may be of value in cardiac patients with superimposing inflammatory diseases such as rheumatoid arthritis.

Since inflammation can affect drug targets such as drug metabolizing enzymes, transporters and effector molecules, thereby, skewing the fate of the drug in the body, we were interested to characterize the effect of inflammation on those target molecules. Inflammation resulted in significant alterations of several uptake and efflux transporters in the rat liver, heart and kidney. These findings may provide an insight into the effect of inflammation on important drug targets

and modulators of disease pathogenesis. We used real time RT-PCR, a fast and efficient technique used to detect and quantify expression of target genes. Since careful selection of the most suitable housekeeping genes is an important factor in having reliable results, we evaluated the suitability of using three housekeeping genes in a chronic inflammation model, Pre-AA. The suitability ranking of the three genes was 18s rRNA, GAPDH then β-actin.

Inflammation is the missed ring in the chain of therapy. Inflammation downregulates drug metabolizing enzymes, alters plasma protein concentrations, alters drug transporter expression and downregulates drug effector molecules. Hence, it should be considered in the design of disease management guidelines. The research presented in this thesis adds to the inflammation-drug interaction field important findings that indeed will set the stage for further in-depth studies to determine how clinically significant these interactions and to explain the reason behind therapeutic failures.

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