Homocysteine Mediated Oxidative Stress and Endothelial Dysfunction

A Thesis

Submitted to the Faculty

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By

Vathsala Edirimanne

In partial fulfillment of the

Requirement for the Degree

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Abstract

Hyperhomocysteinemia or an elevation of blood homocysteine (Hcy) levels is a risk factor for cardiovascular disorders. In this study, the NADPH oxidase involvement in Hcy-induced superoxide anion accumulation in the aorta, which led to endothelial dysfunction during hyperhomocysteinemia was investigated. Hyperhomocysteinemia was induced in rats fed a high-methionine diet. The NADPH oxidase activity, the levels of superoxide, peroxynitrite and expression of NADPH oxidase $p22^{phox}$ subunit were markedly increased as well as the impairment of endothelium-dependent relaxation in aortas isolated from hyperhomocysteinemic rats. Administration of an NADPH oxidase inhibitor, apocynin was able to reverse those effects. Transfection of human vascular cells with $p22^{phox}$ siRNA inhibited the NADPH oxidase dependent Hcy-induced superoxide anion production. In conclusion, Hcy-stimulated superoxide anion production in the vascular wall is mediated through the activation of NADPH oxidase, which leads to endothelial dysfunction during hyperhomocysteinemia.

Key words: homocysteine, oxidative stress, NADPH oxidase, vascular cells

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Last but not least I would like to thank my husband, Gamika Prathapasinghe for the assistance and encouragement given me through out the research period.

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List of Abbreviations

Abbreviation	Name
$^1O_2$	Singlet oxygen
5-MTHF	5-methyl tetrahydrofolate
ATP	Adenosine triphosphate
BHMT	Betaine homocysteine methyl transferase
BSA	Bovine serum albumin
Ca^2+	Calcium
$CaCl_2$ $2H_2O$	Calcium chloride
CAD	Coronary artery disease
CBS	Cystathionine beta synthase
cDNA	Complementary DNA
cGMP	Cyclic guanylate monophosphate
CVD	Cardiovascular diseases
DAB - 3, 3	Diaminobenzidine
DEPC H_2O	Diethylpyrocarbonated water
DH_2O	Deionized water
DHE	Dihydroethidium
DNA	Deoxyribonucleic acid
dNTP	2'-deoxynucleoside 5'-triphosphate
DTT	DL-dithiothreitol
ECGS	Endothelial cell growth supplement

ECL	Enhance chemiluminescene reagent
EDHF	Endothelium derived hyperpolarizing factor
EDRF	Endothelium derived relaxing factor
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
eNOS	Endothelial nitric oxide synthase
FAD	Flavin adenine dinucleotide
FBS	Fetal bovine serum
FMD	Flow mediated dilatation
FMN	Flavin mononucleotide
GAPDH	Glyceraldehydes 3-phosphate dehydrogenase
GTPase	Guanylate triphosphate enzyme
H_2O_2	Hydrogen peroxide
Hcy	Homocysteine
HEPES	4-(2-hydroxyethyl)-1 piperazineethanesulfonic acid
HEPES - KH	Krebs Henseleit buffer
HHcy	Hyperhomocysteinemia
HOCl	Hyperchlorus acid
h	Hour
HUVEC	Human umbilical vein endothelial cells
iNOS	Inducible nitric oxide synthase
ITS	Insulin sodium selinite

KCl	Potassium chloride
KH_2PO_4	Potassium phosphate dibasic
kDa	Kilodalton
$MgCl_2$	Magnesium chloride
$MgSO_47H_2O$	Magnesium sulfate heptahydrate
min	Minute
M – MLV-RT	Maloney murine leukemia virus reverse transcriptase
mRNA	Messenger ribonucleic acid
MS	Methionine synthase
MTHFR	Methyltetraydrofolate reductase
Na_2HPO_4	Disodium biphosphate
NaCl	Sodium chloride
NADPH	Nicotinamide adenine dinucleotide phosphate
NADPH oxidase	Nicotinamide adenine dinucleotide phosphate oxidase
NaH_2PO_4	Sodium phosphate
$NaHCO_3$	Sodium bicarbonate
$NaNO_2$	Sodium nitrate
NBT	Nitrobluetetrasolium
NH_4^+	Ammonium ion
nNOS	Neuronal Nitric oxide synthase
$NO^{\cdot}$	Nitric oxide
NO_2	Nitrogen dioxide

NOS	Nitric oxide synthases
$O_2^{\cdot}$	Superoxide
O_3	Ozone
$OH^{\cdot}$	Hydroxyl
$ONOO^{-}$	Peroxynitrite
PBS	Phosphate buffered saline
PMSF	Phenylmethanesulphonylfluoride
PCR	Polymerase chain reaction
RNS	Reactive nitrogen species
RT	Reverse transcriptase
RNAs	Ribonucleic acids
RLU -	Relative light unit
RNA	Ribonucleic acid
RNasin	Ribonuclease inhibitor
$RO^{\cdot}$	Alkoxyl
$RO_2^{\cdot}$	Peroxyl
ROS	Reactive oxygen species
RT- PCR	Reverse transcript polymerase chain reaction
SAH	S-adenosylhomocysteine
SAM	S-adenosylmethionine
SAM synthase	S-adenosylemethionine synthase
SDS	Sodium dodecylsulphate

SH	Sulfhydryl
SO_4^+	Sulfate ion
SOD	Superoxide dismutase
TBS	Tris-buffered solution
TBST	Tris-buffered solution with 0.1% Tween-20
Tris- HCl	Tris-hydrochloric acid
VSMC	Vascular smooth muscle cells

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

Hyperhomocysteinemia (HHcy), a condition of elevated blood homocysteine (Hcy) levels, is an independent risk factor for cardiovascular diseases (CVD) (Clarke et al. 1991; McCully 1996). Although the precise mechanisms of Hcy-induced CVD remain to be investigated, oxidative stress and endothelial dysfunction are considered to be two major possible mechanisms (Au-Yeung et al. 2004; Weiss et al. 2002b). A previous study done by our laboratory demonstrated that superoxide ($O_2^{\cdot-}$) levels were elevated in the aortas of hyperhomocysteinemic rats (Au-Yeung et al. 2004). However, the mechanism by which Hcy induces $O_2^{\cdot-}$ production in the aortic tissue was not addressed. Furthermore, the causal relationship between increased $O_2^{\cdot-}$ production and the impairment of endothelium-mediated vascular relaxation remains to be clarified.

1.1.1 Objectives of the Study

The general objective of my study was to investigate the mechanisms of Hcy-induced oxidative stress and endothelium-mediated dysfunction in the rat aorta. Specifically we aimed to;

1. Determine whether the NADPH oxidase system is involved in mediating the Hcy-induced oxidative stress. The main $O_2^{\cdot-}$ producer in aorta during pathological situations is the nicotinamide adnenine dinucleotide phosphate oxidase (NADPH oxidase) system (Bendall et al. 2007; Mueller et al. 2005; Oelze et al. 2006). Therefore, the basis of the research was focused on this $O_2^{\cdot-}$ producing system. By subjecting rat aortas to hyperhomocysteinemic conditions and subsequently exposing them to an NADPH oxidase inhibitor, apocynin, objective was to

determine the extent to which the NADPH oxidase system is involved in Hcy mediated oxidative stress.

2. Determine whether Hcy has an effect on NADPH oxidase subunit expression. If the NADPH oxidase system was found out to be significantly involved in Hcy mediated $O_2^{\cdot-}$ production in aorta, the second objective was to determine whether the observed effect was associated with NADPH oxidase enzyme subunit upregulation.
3. Determine the causal relationship between NADPH oxidase mediated $O_2^{\cdot-}$ level and the impaired endothelium-dependent vascular relaxation during HHcy. Under this objective we planned to determine if NADPH oxidase mediated $O_2^{\cdot-}$ could have a reducing effect on the bioavailability of nitric oxide ($NO^{\cdot}$), the main factor in endothelium-dependent vascular relaxation.
4. Examine the effects of Hcy on eNOS-mediated $NO^{\cdot}$ production.

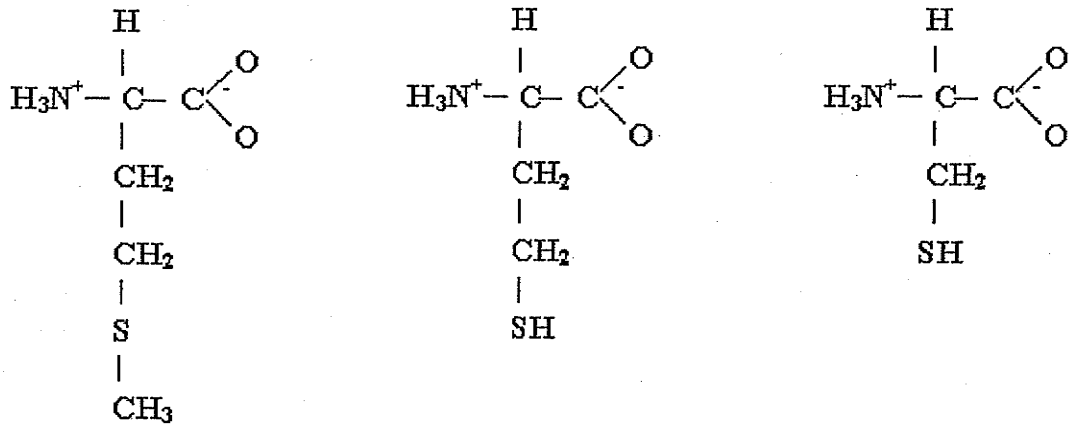
1.1.2 Hypotheses

This study was based on two hypotheses. The first hypothesis was that Hcy-induced oxidative stress was due to the upregulation of the NADPH oxidase system. The second hypothesis was that $O_2^{\cdot-}$ produced by the NADPH oxidase system was responsible for endothelial dysfunction during HHcy.

1.2 Literature Review

1.2.1 Homocysteine Metabolism

Homocysteine (Hcy) has been studied extensively for over 30 years for its unique involvement in an increasing number of human diseases. Hcy is a sulfhydryl group-containing amino acid synthesized as an intermediate product during methionine



Methionine **Homocysteine** **Cysteine**

Figure 1.1 Structures of Methionine, Homocysteine and Cysteine (Brosnan and Brosnan 2006).

metabolism. It contains a free sulfhydryl (-SH) group attached to its side chain and a structure similar to cysteine with an additional methyl group (Figure 1.1). In general, Hcy does not participate in protein synthesis under biological conditions but it has the ability to interfere with the incorporation of methionine during protein synthesis (Jakubowski 2003). Under *in vitro* conditions, however, derivatives of Hcy can act as methionine homologues and participate in a limited manner in protein synthesis (Jakubowski 2003). A non-significant amino acid in terms of protein synthesis, Hcy plays an important role in the methionine metabolic pathway (Figure 1.2). The immediate dietary precursor for Hcy is methionine. Methionine is an essential amino acid and its metabolism leads to the synthesis of cysteine, taurine and glutathione. To understand the role of Hcy in the biological systems, it is important to understand its involvement in the methionine metabolic pathway. Methionine metabolism consists of three major pathways, namely, transmethylation, transsulfuration and remethylation. The transmethylation pathway converts dietary methionine into Hcy after several biochemical reactions. The transsulfuration pathway catabolizes Hcy into cysteine, therefore, assisting Hcy removal from the cells. The remethylation pathway is capable of re-synthesizing methionine from pre-formed Hcy. This process is important both in preventing the excess accumulation of Hcy as well as replenishing the methionine pool.

1.2.1.1 Transmethylation

The first reaction of the methionine transsulfuration pathway is the combination of methionine and adenosine triphosphate (ATP) to form S-adenosylmethionine (SAM). This reaction is catalyzed by S-adenosylemethionine synthase (SAM synthase) or

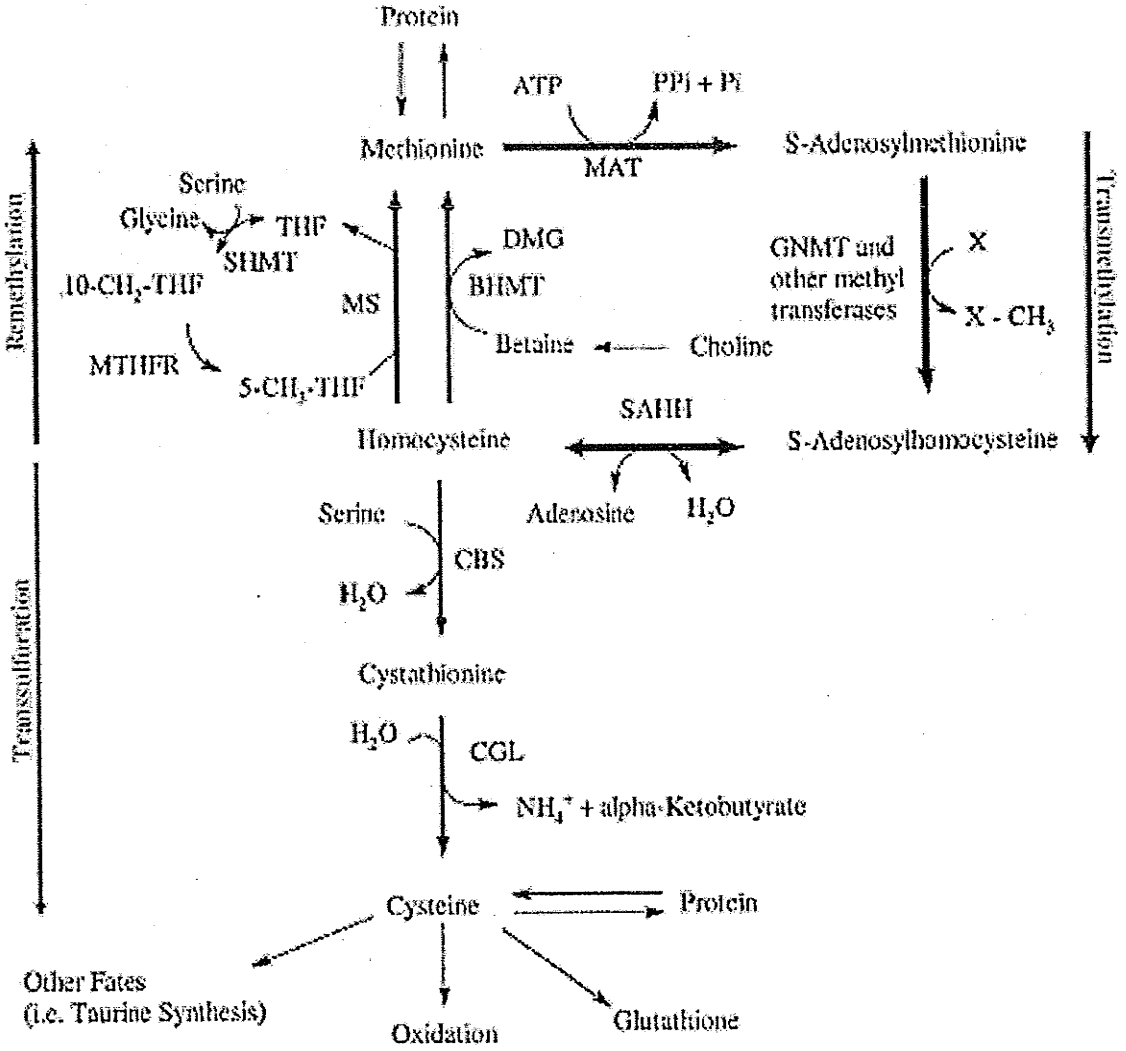


Figure 1.2 Homocysteine metabolism
MAT-methionine adenosyltransferase, **CGL**-cystathionine gamma lyase, **CBS**-cystathionine beta synthase, **GNMT**-Glysine N-methyl transferase, **SAHH**-S-adenosylhomocysteine hydrolase, **BHMT**-betaine homocysteine methyl transferase, **DMG**-Dimethylglycine, **MS**-methionine synthase, **MTHFR**-methyltetrahydrofolate reductase, **THF**-tetrahydrofolate, **SHMT**-Serine hydroxyl methyl transferase, **5-10-CH_2 THF**-5, 10 methylene tetrahydrofolate, **5-CH_3 THF**-5 methyl tetrahydrofolate (Brosnan and Brosnan 2006).

methionine adenosyltransferase (MAT) (Mato et al. 1997). MAT exists in three isoforms in mammalian tissues. MAT-I and MAT-III are liver specific isozymes. MAT-II is a non-liver specific isozyme (Okada et al. 1981; Sullivan and Hoffman 1983). Due to this tissue specificity and the different half maximal value (K_m) of MAT isoforms for methionine, the tissue content of SAM is a function of both the organ concerned and the availability of methionine. SAM is a high energy sulfonium compound, which can transfer its energized methyl group to a large number of methyl acceptors such as DNA, RNA, protein, etc. SAM is the methyl donor in about 115 methyltransferase reactions in human (Scott et al. 1994). SAM dependent transmethylation reactions are essential for a diverse range of physiological functions, each of which are catalyzed by specific transmethylase enzymes (De La Haba and Cantoni 1959). All the transmethylases are inhibited by the product S-adenosylhomocysteine (SAH) (Finkelstein 1998). During the transfer of a methyl group, SAM is converted into SAH. SAH is hydrolyzed to Hcy and adenosine in a reversible reaction catalyzed by S-adenosylhomocysteine hydrolase (De La Haba and Cantoni 1959). Hcy is positioned at a critical junction in the methionine metabolic pathway. At this point Hcy can either enter into the remethylation pathway and can be remethylated into methionine or Hcy can enter into the transsulfuration pathway and irreversibly catabolized into cysteine.

1.2.1.2 Transsulfuration

The first reaction of the transsulfuration pathway is the conversion of Hcy to cystathionine. Hcy is combined with serine by the catalytic effect of cystathionine beta synthase (CBS) (Banerjee et al. 2003). This reaction is irreversible. From this point onwards the reaction is committed towards the removal of Hcy by converting to cysteine

(Finkelstein 1998). The irreversible nature of this reaction and the absence of Hcy in the mammalian diet makes methionine a dietary essential amino acid (Banerjee et al. 2003). In the second reaction, cystathionine is catabolized into cysteine, ammonium ion (NH_4^+) and alpha-ketobutyrate by cystathionine gamma lyase (CGL). Cysteine derived by this reaction is either incorporated into protein, utilized for glutathione synthesis, taurine synthesis or oxidated to sulfate ion (SO_4^+) and water.

1.2.1.3 Remethylation

Hcy, after entering into the remethylation pathway, is converted back to methionine. Hcy acquires an additional methyl group through folate dependent or folate independent reactions. Hcy can accept a methyl group either from N^5-methyltetrahydrofolate (5-MTHF) or from betaine. The source of the primary methyl donor in the remethylation pathway depends on the type of tissue (Delgado-Reyes et al. 2001). In tissues such as liver, kidney and intestine, both sources act as methyl group donors. However, in other tissues only 5-MTHF acts as the methyl group donor because those tissues lack the enzyme betaine homocysteine methyl transferase (BHMT) (Delgado-Reyes et al. 2001).

The enzyme that connects methionine metabolism and folate metabolism is methionine synthase (MS). MS needs vitamin B_{12} (cobalamin) as a cofactor (Stipanuk 2004). 5-MTHF acts as the substrate for MS (Matthews 2001). The methyl group is first transferred from 5-MTHF to the cobalamin group of vitamin B_{12} to form methylated cobalamin (Matthews 2001). Methionine is generated as a result of the methyl group transfer from methylated cobalamin to Hcy. Vitamin B_{12} is essential for the forward drive of this reaction. Deficiency of vitamin B_{12} results in accumulation of intracellular 5-MTHF. This is termed as 'folate trapping'(Scott and Weir 1981). The catalytic enzyme of

the folate independent remethylation pathway is BHMT (Finkelstein et al. 1982). Unlike in humans where BHMT is found in the liver, kidney and intestines, in rats, BHMT activity is found only in the liver (Finkelstein et al. 1982). Betaine, obtained from dietary sources or from the catabolism of choline, is the methyl group donor in this reaction (Sakamoto et al. 2002; Zeisel et al. 2003).

1.2.2 Regulatory Mechanisms of Homocysteine Metabolism

The fate of Hcy depends on several factors, i.e. the level of methionine in the diet, SAM level and the type of cells in which methionine metabolism takes place (Mudd et al. 1965). High SAM level acts as an allosteric inhibitor for methylenetetrahydrofolate reductase (MTHFR) of the remethylation pathway, which catalyzes the formation of 5-methyl tetrahydrofolate (5-MTHF). 5-MTHF, as stated previously, is one of the two methyl donors for the remethylation of Hcy to methionine (Finkelstein and Martin 1984). High SAM level inhibits the production of 5-MTHF. Therefore, the presence of high SAM prevents Hcy from entering into the remethylation pathway.

High SAM level also acts as an allosteric activator for CBS of the transsulfuration pathway (Finkelstein et al. 1975; Stabler et al. 2002). Only a few organs in the body, such as the liver, kidney, pancreas, brain, adipose tissue and small intestine, have the capability to synthesize CBS (Finkelstein 1990; Mudd et al. 1965). However, brain and adipose tissue have an incomplete transsulfuaration pathway due to a lack of cystathionase (Finkelstein 1998). In the tissues containing the complete transsulfuration pathway, the presence of a high level of SAM leads to the forward drive of the transsulfuration pathway. Therefore, the presence of a high level of SAM favours Hcy into entering the transsulfuration pathway. However, other organs such as blood vessels

in which CBS does not exist in the active form, high SAM does not have this effect. Rather, in vascular tissues, a high SAM level leads to transient accumulation of Hcy.

Hcy level in the plasma depends on the half maximal values (K_m) of the enzymes of the transulfuration and transmethylation pathways. Generally the K_m for their substrates of the enzymes in the transsulfuration pathway are higher than the K_m for their substrates of the enzymes in the transmethylation pathway thus, saturability of the enzymes in the transsulfuration pathway is higher than that of the enzymes in the remethylation pathway. Thus, after dietary methionine overload the Hcy level in blood is transiently elevated (Stipanuk 2004).

The changes in dietary methionine availability and the resulting intracellular methionine level affect the rate of SAM synthesis, which in turn, will determine the pathway Hcy takes at the remethylation and the transsulfuration junction (Selhub 1999).When the diet contains a basal methionine level, Hcy cycle through the remethylation pathway about 1.5-2.0 times before directed towards the transsulfuration pathway (Eloranta et al. 1990). When dietary methionine content is half the basal level, the cycling of Hcy via the remethylation pathway increases by twofold. Conversely, when the dietary methionine level is high, Hcy cycling through the remethylation is reduced by about 1.5 fold (Eloranta et al. 1990). In other tissues where CBS is not present, excess Hcy is directed towards the remethylation pathway or exported out into the circulation.

1.2.3 Cellular Homocysteine Transportation

Hcy is synthesized intracellularly. Hcy accumulation is highly toxic to cells therefore, the intracellular Hcy concentration is always maintained at a low level (Huang et al. 2007; Hultberg and Hultberg 2007). This is especially important in tissues that do not have a

transsulfuration pathway. For example, heart and vascular cells are unable to metabolize Hcy into cysteine because these tissues do not express CBS (Chen et al. 1999). Intracellular Hcy concentration is strictly regulated by export mechanisms (Ueland and Refsum 1989). Under normal conditions cells export a considerable amount of Hcy to the circulation (Hultberg et al. 1998). It has been found that export of Hcy from endothelial cells is higher than that of hepatoma cells (Hamilton et al. 2001; Hultberg et al. 1998). Furthermore, the uptake of extracellular Hcy into endothelial cells is lower than other types of cells (Hultberg et al. 1998). Elevated intracellular Hcy production leads to increased export of Hcy. Therefore, Hcy export occurs at a balance that reflects intracellular Hcy production and further metabolism (Hultberg et al. 1998). This happens by the immediate export of Hcy out of the cells into the circulation (Jacobsen 1998). The exact mechanism of Hcy export is not clearly identified. However, it has been suggested that Hcy utilizes the same transporters as cysteine, namely, systems XAS, L, ASC and A (Ewadh et al. 1990). It is also believed that the same transport mechanisms are being utilized during Hcy import into the cells during HHcy situations as demonstrated by *in vitro* studies (Budy et al. 2006).

1.2.4 Hyperhomocysteinemia and Cardiovascular Diseases

A total plasma Hcy level above the normal physiological level is termed as hyperhomocysteinemia (HHcy). HHcy can result from either increased production or decreased removal of Hcy. The location of Hcy in the three-way junction of the methionine metabolic cycle renders it susceptible to concentration fluctuations during certain situations.

1.2.4.1 Causative Factors for Hyperhomocysteinemia

1.2.4.1.1 Non Genetic Factors

Several dietary factors such as high methionine intake and/or low folic acid intake, lifestyle factors such as smoking and coffee consumption, deficiencies of vitamin B_6 and vitamin B_{12} and some other factors such as old age and male sex are associated with elevated plasma Hcy levels (Hankey and Eikelboom 1999; Welch and Loscalzo 1998). Dietary methionine is obtained from protein rich food sources such as red meat and cow milk. Excessive intake of these types of food would lead to transient elevation of Hcy levels in blood. Vegetarians have higher plasma Hcy levels than non vegetarians due to the fact that their diet is lacking in vitamin B_{12} (Geisel et al. 2005). Reduced dietary folic acid intake is associated with high plasma Hcy levels and elevated risk for ischemic stroke in the absence of other lifestyle and dietary risk factors (He et al. 2004). Vitamin deficiencies are by far the most common cause for HHcy in the general population (Haynes 2002; Herrmann et al. 1999; Joosten et al. 1993; Naurath et al. 1995). Vitamin deficiencies can occur due to lack of supplement, reduced absorption in the gastrointestinal tract, increased requirement (pregnancy, acquired immunity deficiency syndrome), and interaction with drugs. People who consume vegetarian diets, elderly people, pregnant women, patients with renal failure, malabsorption diseases such as irritable bowel syndrome are in the high risk group for vitamin deficiency mediated HHcy (Castro et al. 2006). Folate deficiency is the most common vitamin deficiency. Vitamin B_{12} deficiency occurs frequently in elderly people. Malabsorption of vitamin B_{12} affects 30-40% of the elderly population (Beitz et al. 2002; Herrmann et al. 2005; Obeid et al. 2004; Selhub et al. 1993).

Serum creatinine level is one of the strongest determinants of the fasting Hcy levels which reflects the effect of renal function on plasma Hcy concentration (Arnadottir et al. 1996; Bostom and Culleton 1999). Creatinine synthesis is a SAM-dependent transmethylation reaction and renal failure is accompanied by elevated total plasma Hcy concentrations (Bostom and Culleton 1999). Renal failure patients have higher levels of Hcy compared with normal individuals due to impaired remethylation and transsulfuration pathways (Herrmann et al. 2001; Obeid et al. 2005).

Large population studies have established that the male gender is associated with HHcy (Jacques et al. 1999; Nygard et al. 1995). Men have 28% higher plasma/serum Hcy concentration than women (Battezzati et al. 2007). Remethylation and transsulfuration rates were found to be higher in pre-menopause women than in men (Fukagawa et al. 2000). After menopause there is a rise in plasma Hcy levels probably due to the fluctuation of estrogen concentrations (Brattstrom et al. 1985). The reduced muscle mass associated with the female compared to the male may also explain the reduced plasma Hcy levels which are reflected by low creatinine levels (Jacques et al. 1999; Lussier-Cacan et al. 1996).

Plasma Hcy concentration gradually increases with age. It is almost double the childhood concentrations in elderly people (Refsum et al. 2004b). Several factors such as reduced vitamin bioavailability and decline of renal function may be contributing factors. Other factors such as smoking, lack of physical activity, coffee consumption and alcoholism can have a marked influence on higher Hcy levels (Lievers et al. 2003). Pregnancy and a low consumption of alcohol has a reducing effect on plasma Hcy levels. Traditional cardiovascular risk factors such as elevated cholesterol levels and hypertension have a

positive correlation with plasma Hcy levels (Lievers et al. 2003). Certain drugs and chemicals can affect Hcy metabolism such as theophylline, carbamazepine and valproate. Theophylline is a vitamin B_6 antagonist whereas valproate has antifolate effects. Therefore, HHcy resulting from these drugs is secondary to vitamin deficiencies (Karabiber et al. 2003).

1.2.4.1.2 Genetic Factors

The most common genetic factor leading to elevated Hcy levels is the presence of a thermo labile variant of MTHFR. This mutant has a 70% reduced enzyme activity (677C→T mutation). 5%-14% of the general population is homozygous for this mutation (Frosst et al. 1995). A recent meta analysis showed that homozygous MTHFR defect is associated with a 20% higher risk for degenerative vascular disorders (Klerk et al. 2002; Meleady et al. 2003). A heterozygous MTHFR defect leads to a 25% elevation of Hcy in the circulation. Homocystinuria due to a CBS defect was the most common cause for severe HHcy with incidences estimated at 1:6400 (Refsum et al. 2004a). Inadequate CBS activity leads to the accumulation and export of Hcy into the circulation leading to HHcy.

1.2.4.2 Plasma Homocysteine Forms and Concentrations during Mild, Moderate and Severe Hyperhomocysteinemia

The name 'Homocysteine' was first introduced by the discoverer of Hcy, Du Vigneaud in 1932 (Mudd et al. 2000). The concentration of total plasma Hcy is referred to as 'homocysteine' as a convenient and simple form of identification. This term generally referred to both the reduced (sulfhydryl) and oxidized (disulfide) forms of Hcy, which can be misleading. Most of the reduced form of Hcy entering into the circulation

becomes oxidized within a few minutes (Mansoor et al. 1993a; Mansoor et al. 1992). The total plasma Hcy is comprised of both the reduced and oxidized forms of Hcy. The reduced form of Hcy is generally known as 'homocysteine' and it has a free -SH group. There can be three forms of oxidized Hcy. Hcy can combine with another Hcy molecule (auto-oxidation) and form 'homocystine', bind with a cysteine molecule and form homocysteine-cysteine mixed disulfide molecule or it can oxidize the -SH groups on proteins such as serum albumin to form 'protein-bound homocysteine mixed disulfides' (Figure 1.3). The concentrations of low molecular weight homocystine and homocysteine-cysteine mixed disulfide in serum is 5%-10% of the total Hcy concentration, with protein-bound homocysteine mixed disulfides making up to about 80%-90 % of the total Hcy concentration (Refsum et al. 1985; Wiley et al. 1988). The major carrier of Hcy in human serum is serum albumin (Refsum et al. 1985). The oxidized form of Hcy comprises more than 98% of the total Hcy concentration (Mansoor et al. 1992). The reduced or free form of Hcy is only less than 2% in normal to mild Hcy people (Mansoor et al. 1995; Mansoor et al. 1993b; Ueland 1995). However, homocystinuric patients have much higher (5%-20%) reduced Hcy levels (Mansoor et al. 1993b). The normal physiological Hcy concentration in blood in humans is 5-15 μmol /L. Any level above this is considered as a hyperhomocysteinemic situation. Concentrations between 16-30 μmol /L is considered as mild HHcy, 31-100 μmol /L as moderate HHcy and concentrations above 100 μmol /L is termed as severe HHcy (Refsum et al. 2004b). Measuring total Hcy levels after methionine overload can be utilized as a diagnostic procedure for mild abnormalities in Hcy metabolism (Bostom et al. 1995).

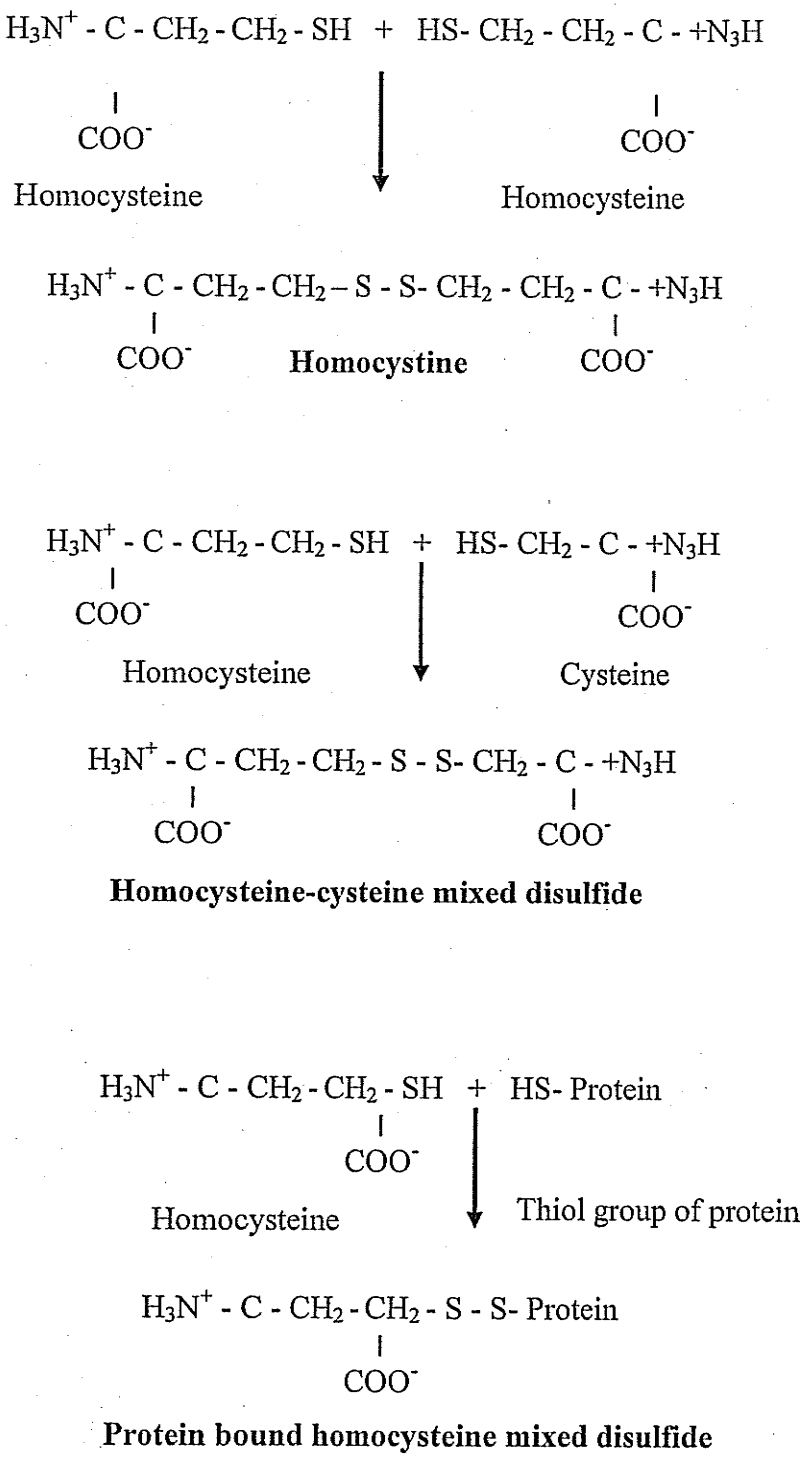


Figure 1.3 Oxidized Forms of Homocysteine in Plasma
(Brosnan et al. 2004)

1.2.4.3 Cardiovascular Diseases

Cardiovascular diseases (CVD) are the leading health hazard worldwide. CVD is the number one killer in North America according to the American Heart Association (Thom et al. 2006). The established traditional risk factors for cardiovascular diseases are: age, male sex, hypercholesterolemia, genetic factors, hypertension, obesity, diabetes, cigarette smoking, and lack of exercise. (Schauffler et al. 1993; Wilson 1994).

The relationship between HHcy and cardiovascular diseases first identified by the American pathologist McCully in 1969 (McCully 1969). He observed that in patients with homocystinuria (a genetic disorder associated with CBS deficiency) severe HHcy, premature atherothrombotic disease and early death prevailed. Without treatment an estimated 50% of the patients will have a complication in their vascular system before the age of 30 years (Finkelstein et al. 1969; Mudd et al. 1964; Mudd et al. 1985). Even though the observational relationship between severe HHcy and atherosclerosis in this disorder was quite apparent, noone has proposed a link between the two occurrences until 1975 (McCully and Wilson 1975). In 1976 Wilcken and Wilcken confirmed that not only severe HHcy but also moderate HHcy has an elevated incidences of atherosclerotic vascular disorders (Wilcken and Wilcken 1976). Since then a vast number of studies were done to further establish the link between vascular diseases and HHcy. The first population based study on Hcy was the 'Hordaland homocysteine study' which started in 1991 (Refsum et al. 2006). It involved about 18,000 men and women and was conducted in Hordaland county, western Norway. The first phase of the study conducted from 1992-93 was based on participants of age 40-67 years old (Refsum et al. 2006). A follow up study was conducted in 1997-99. The final conclusion of this study was that subjects with

elevated plasma Hcy levels have a high risk of cardiovascular morbidity or cardiovascular mortality (Refsum et al. 2006).

The significance of the association of CVD and total plasma Hcy levels increases with Hcy concentrations of more than 15 μmol/L (Refsum et al. 2006). The meta analysis done by Boushey et al. in 1995 of a total of 27 studies including population based case control studies and prospective studies revealed several key aspects of elevated plasma Hcy levels and CVD (Boushey et al. 1995). This study concluded that elevated plasma Hcy level is an independent and graded risk factor for CVD (Boushey et al. 1995). Furthermore, odds ratios for 5 μmol/L increase in plasma tHcy with coronary artery disease ranged from 1.5 to 1.8 for men and women. Indicating that men have a higher risk for coronary artery disease with elevations of plasma Hcy levels (Boushey et al. 1995). Coronary artery disease was attributable to the elevated plasma Hcy levels in 10% of the subject population (Boushey et al. 1995). However, the causal connection between HHcy and CVD still needs to be proven beyond reasonable doubt by showing that CVD incidences are reduced by lowering of Hcy levels possibly by vitamin therapy (folic acid). The lowering of Hcy by 25% is associated with 10% lower risk of CVD or 20% less stroke (Herrmann 2006). On going prospective meta analysis of Hcy lowering trials might provide a stronger link between HHcy and intervention therapy. The collective data from more than 80 clinical trials involving more than 11,000 patients indicated that HHcy is an independent risk factor for peripheral heart disease, myocardial infarction and stroke (Clarke et al. 1991; Malinow 1996; Verhoef et al. 1996; Wilcken and Wilcken 1976). Overall approximately 30 epidemiological studies published during the last ten years show that even mild HHcy is associated with vascular diseases (Boers et al. 1985;

Brattstrom et al. 1984; Malinow 1996; Malinow et al. 1989). However, it is still debatable whether Hcy is an independent risk factor for CVD or whether it is a bio marker for CVD. One reason for this is that in major vitamin intervention trials such as HOPE 2 trail did not show a significant reduction of CVD dispite the vitamin B therapy (Herrmann 2006). Identifying Hcy mechanism of action during CVD would lead to a more definitive unserstanding of Hcy involvement in CVD.

1.2.5 Mechanisms and Systems Involved in Homocysteine Associated Cardiovascular Complications

Several possible mechanisms have been proposed over the years to explain the mechanisms of action of Hcy in CVD. These include oxidative stress created by elevated reactive oxygen species (ROS) production, reduction of nitric oxide bioavailability, endoplasmic reticulum-stress, dysfunction of endothelium mediated vasodilation, chemokine activation, adhesion molecule activation, low density lipoprotein oxidation, lipid peroxidation, vascular smooth muscle cell proliferation, abnormalities in platelet function leading to thrombosis, direct endothelial cell injury and activation or repression of gene transcription (Au-Yeung et al. 2004; Outinen et al. 1999; Wang et al. 2002; Weiss et al. 2002a; Weiss et al. 2002b).

1.2.5.1 Oxidative Stress

The normal physiological environment is a balanced redox environment. The intracellular environment is predominantly a reducing environment due to the presence of glutathione, the body's major anti-oxidant. During biochemical reactions, a certain amount of pro-oxidative reactive oxygen species (ROS) are formed. However, glutathione can

effectively reduce these agents and maintain the redox balance. During pathological conditions when a large amount of ROS are formed, the pro-oxidative state overrides the anti-oxidative capability of glutathione and a redox imbalance is created (Fortuno et al. 2005). This state is generally known as oxidative stress.

1.2.5.1(a) Reactive Oxygen Species

ROS are important mediators of oxidative stress or cell signaling (Groemping and Rittinger 2005). ROS are oxygen derived small molecules that are highly reactive and constantly being synthesized and catabolized from either endogenous or exogenous systems (Szasz et al. 2007). ROS are produced by the gradual reduction of molecular oxygen. They can be either unstable free radicals such as superoxide ($O_2^{\cdot-}$), hydroxyl ($OH^{\cdot}$), peroxyl ($RO_2^{\cdot}$) or alkoxyl ($RO^{\cdot}$) or relatively stable non free radicals that can be converted into free radicals such as hyperchlorous acid (HOCl), ozone (O_3), singlet oxygen ($^1O_2$) and hydrogen peroxide (H_2O_2). Nitrogen containing ROS such as nitric oxide ($NO^{\cdot}$) are called reactive nitrogen species (RNS) (Bedard and Krause 2007). The generation of ROS is a cascade of reactions that starts with the production of $O_2^{\cdot-}$ which is rapidly converted into H_2O_2 either spontaneously at low pH or by the catalytic activity of superoxide dismutase (SOD) (Fridovich 1997). $O_2^{\cdot-}$ is rapidly removed by the scavenging action of SOD which has several isoenzymes located in the mitochondria, cytoplasm, and extracellular compartments (Pacher et al. 2007). Another fate of the ROS cascade is that $O_2^{\cdot-}$ react with $NO^{\cdot}$ to form a more reactive RNS called peroxynitrite ($ONOO^-$) (Beckman 2002; Beckman et al. 1994; Beckman and Koppenol 1996).

ROS can react with inorganic molecules such as $NO^{\cdot}$ as well as a large number of organic biological molecules such as proteins, lipids, carbohydrates and nucleic acids which lead

to the irreversible alteration of their structure and thereby, their function. ROS have being identified as one of the major sources of damage to biological organisms. ROS are involved in the aging process, as a component of innate immunity and also as cell signaling molecules which are responsible for a large number of reversible regulatory processes in the body (Harrison 1997; Lavrovsky et al. 2000; Rajagopalan et al. 1996b; Szasz et al. 2007). The generation of ROS can occur as a by-product of the function of several enzymatic systems such as mitochondrial oxidative phosphorylation, cytochrome P450-detoxifying reactions, peroxysomal system, nitric oxide synthase uncoupling, xanthine oxidase system, cyclooxygenases and lipoxygenases (Touyz and Schiffrin 2004) (Figure 1.4). The presence of enzyme systems as the primary $O_2^{\cdot-}$ producers came to light after the discovery of phagocytic cell (neutrophils, macrophages and eosinophils) nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase), a professional $O_2^{\cdot-}$ producing enzyme system ($O_2^{\cdot-}$ is produced as the primary product by this enzyme system) (Szasz et al. 2007). Discoveries of new tissue specific NADPH oxidase systems that also synthesized $O_2^{\cdot-}$ as their primary product, led towards numerous studies that investigated the structure and function of NADPH oxidases.

1.2.5.1(b) Nicotinamide Adenine Dinucleotide Phosphate Oxidase

NADPH oxidase utilizes reduced nicotinamide adenine dinucleotide phosphate (NADPH) as the substrate. While converting NADPH to its oxidized form NADP, the enzyme can transfer the removed electron across biological membranes to an electron acceptor which is molecular oxygen. The end product of this reaction is a $O_2^{\cdot-}$ anion. Therefore NADPH oxidase can be identified as the primary $O_2^{\cdot-}$ anion producing system (Bedard and Krause 2007).

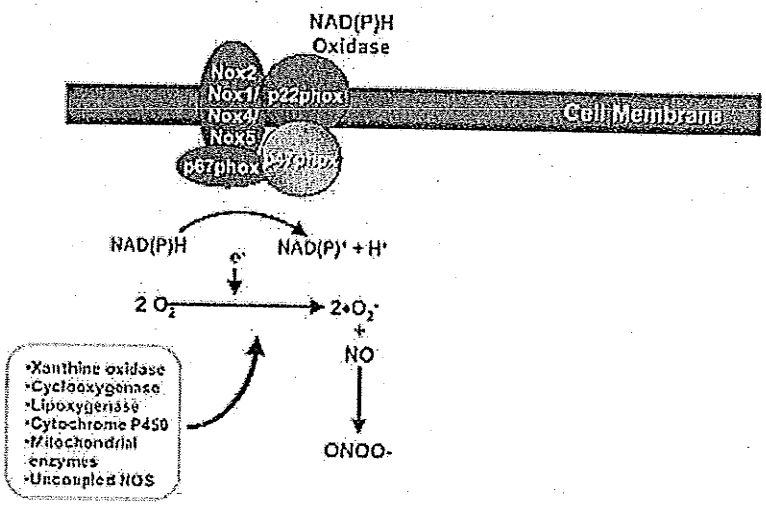


Figure 1.4 Generation of Superoxide $O_2^{\cdot-}$ in Vascular Cells. Many enzyme systems, including NADPH oxidase, xanthine oxidase, and uncoupled nitric oxide synthase (NOS) among others, have the potential to generate reactive oxygen species (ROS). $O_2^{\cdot-}$ acts as a reducing agent, where it donates its extra electron (e−) to form $ONOO^-$ with $NO^{\cdot}$ (Touyz and Schiffrin 2004).

$$2O_2 + NADPH \rightarrow NADP + H^+ + 2O_2^{\cdot -}$$

NADPH oxidase was first characterized as one of the immune defense component of the innate immune system. During pathogenic invasion, phagocytic cells such as neutrophils, eosinophils and macrophages can rapidly release large amounts of $O_2^{\cdot -}$ anions to the extracellular environment or into phagocytic vacuoles (Babior 1984; Robinson and Badwey 1994; Thelen et al. 1993). $O_2^{\cdot -}$ anions are capable of oxidizing microbial cell membranes and causing their eventual destruction. Phagocytic NADPH oxidase is stimulated by bacterial endotoxins and/or exotoxins, chemokines and cytokines (Babior 1999; Vignais 2002). Genetic defects leading to abnormalities of phagocytic NADPH oxidase causes a life threatening disease called chronic granulomatous disease which is characterized by the patients' inability to defend against microbial infections (Dinauer and Orkin 1992; Heyworth et al. 2003). During non-pathogen invasive conditions, phagocytic NADPH oxidase is at a resting phase of inactivation and does not release $O_2^{\cdot -}$ anions. The rapid, sudden release of vast amount of $O_2^{\cdot -}$ anions upon stimulation is termed as 'the oxidative burst' and NADPH oxidase is sometimes referred to as 'the oxidative burst enzyme' (Babior 1984; Thelen et al. 1993). NADPH oxidase is a hetero multi subunit enzyme (Babior 1984) (Figure 1.4). In resting cells the subunits of NADPH oxidase are located in two different cellular compartments, namely the cell membrane and the cytosol. The membrane bound component, a flavocytochrome named cytochrome b558, is the main catalytic core of the enzyme, and it is composed of subunits named $gp91^{phox}$ (recently known as Nox2) and $p22^{phox}$. The 'phox' term is a shortened version for the term - 'phagocytic oxidase'. Cytosolic components are the regulatory components

of the enzyme and are collectively known as 'cytosolic phox' (Babior 1999; Lassegue and Clempus 2003; Robinson and Badwey 1994). These include, $p47^{phox}$, $p67^{phox}$, $p40^{phox}$ and a small guanylate triphosphate enzyme (GTPase) protein Rac (Cross and Segal 2004). Enzyme activation basically occurs through the phosphorylation, translocation and binding of the cytosolic subunits with cytochrome b558 component (Vignais 2002).

It was discovered that NADPH oxidase exists not only in phagocytic cells but almost all of the tissues in the body contain various homologues of this enzyme (Meyer et al. 1999; Shiose et al. 2001). For a time, mitochondrial respiration was thought to be the main contributor to the resting levels of $O_2^{\cdot-}$ anions present in vascular tissues. However, later on this was found to be due to the constitutive activity of vascular NADPH oxidases. Further investigations of the 'non phagocytic NADPH oxidases' revealed that these enzymes, even though structurally similar, have distinct functions than their phagocytic counterparts (Lassegue and Clempus 2003; Li and Shah 2004; Pagano et al. 1997). For example, the NADPH oxidase systems found in vascular cells such as endothelial cells and vascular smooth muscle cells consist of similar membrane bound and cytosolic components but their activation and induction differ from phagocytic NADPH oxidase (Lassegue and Clempus 2003). One of the major differences between the phagocytic and non phagocytic NADPH oxidases is that non phagocytic enzyme systems are constitutivly active as opposed to being inductive (Cai and Harrison 2000; Griendling et al. 2000; Lassegue and Clempus 2003). The vascular NADPH oxidases exist in low levels of activity and release $O_2^{\cdot-}$ constantly under physiological condition. The $O_2^{\cdot-}$ levels produced by vascular NADPH oxidases are approximately 1% that of levels produced by phagocytic NADPH oxidases (Hohler et al. 2000). In contrast to phagocytic

cells, it has been established that a major portion of $O_2^{\cdot-}$ generated by vascular NADPH oxidase is intracellular (De Keulenaer et al. 1998; Landmesser et al. 2002; Paravicini et al. 2002; Park et al. 2004b). Another difference between phagocytic and vascular NADPH oxidase is that vascular NADPH oxidase can be activated acutely or chronically by physical factors such as shear stress or humoral factors such as Angiotensin II. Angiotensin II, a key product of the renin-angiotensin system, which is the best known activator of vascular NADPH oxidases (Castier et al. 2005; Chabrashvili et al. 2003; Griendling et al. 1994; Matsuno et al. 2005; Paravicini et al. 2006; Rajagopalan et al. 1996a). The $gp91^{phox}$ is the main subunit of the catalytic component because binding of other subunits revolve around it. The $gp91^{phox}$ consists of 570 amino acids and has a molecular mass of 65.3 kDa, but runs as a broad smear of approximately 91 kilodaltons (kDa) on SDS/polyacrylamide gels due to the glycosylation of three asparagine residues (Kleinberg et al. 1989; Wallach and Segal 1997). It has a substrate binding domain for NADPH and a cofactor binding domain for flavin adenine dinucleotide (FAD) (Groemping and Rittinger 2005; Rajagopalan et al. 1996b). The N-terminal of $gp91^{phox}$ has two heme binding structures and the location is trans-membrane. The C-terminal is folded into the cytoplasm and contains FAD and NADPH binding sites (Groemping and Rittinger 2005; Rajagopalan et al. 1996b). Once the oxidase is activated, electrons are transferred from the cytoplasmic NADPH to FAD to two heme structures and to molecular oxygen located in the extracellular compartment (Groemping and Rittinger 2005; Rajagopalan et al. 1996b). Five $gp91^{phox}$ homologues have been isolated in mammals so far. These are NOX1, NOX2, NOX3, NOX4 and NOX5 (Banfi et al. 2003; Cheng et al. 2001; Shiose et al. 2001; Suh et al. 1999). NOX1 and NOX4 are both found

in vascular cells. NOX1 is located in vascular smooth muscle cells and NOX4 is predominantly located in endothelial cells (Ago et al. 2004; Lassegue et al. 2001). NOX1 is the first $gp91^{phox}$ homologue to be recognized (Touyz et al. 2002). NOX1 and NOX4 are more or less identical to $gp91^{phox}$ in size and structure. NOX1 and NOX4 shares a 88% and 68% identity in the NADPH binding site with $gp91^{phox}$, respectively (Lambeth et al. 2000). They both generate $O_2^{\cdot-}$ by transferring electrons through FAD and heme containing groups from NADPH to molecular oxygen (Lambeth 2004; Martyn et al. 2006). Binding with membrane bound subunit $p22^{phox}$ is essential for both NOX1 and NOX4 for activation (Ambasta et al. 2004; Lambeth 2004; Takeya et al. 2003).

NOX1 appears to require the cytosolic subunits ($p47^{phox}$ and $p67^{phox}$) for activation (Banfi et al. 2003; Park et al. 2004a). NOX4 appears not to require cytosolic subunits for activation (Martyn et al. 2006).

The $p22^{phox}$ is a non-glycosylated protein and contains two to four transmembrane segments. The C-terminal cytoplasmic end contains a proline rich region which acts as the anchoring site for $p47^{phox}$. The $p22^{phox}$ contains 195 amino acids and has a molecular mass of 22 kDa (Brandes and Kreuzer 2005).

The $p47^{phox}$ has 390 amino acid residues and a molecular weight of 44.7 kDa (Groemping and Rittinger 2005). The $p47^{phox}$ is activated by the phosphorylation of its serine residues (el Benna et al. 1994). Upon phosphorylation, $p47^{phox}$ translocates towards the cell membrane and binds with the anchoring region of $p22^{phox}$ (Groemping et al. 2003; Nakanishi et al. 1992). The $p67^{phox}$ contains 526 amino acid residues with a molecular weight of 59.8 kDa (Groemping and Rittinger 2005).

1.2.5.2 Hyperhomocysteinemia Mediated Endothelial Dysfunction

1.2.5.2 (a) Endothelium Regulated Vascular Function

Endothelium is a monolayer of epithelial cells that covers the internal (luminal) surface of vessels. A healthy endothelium is defined as optimally placed and able to respond to various physical and chemical stimuli by the production of large a number of biological factors that mediate vascular tone and resistance to thrombus formation, molecular adhesion and vascular smooth muscle cell proliferation (Feletou and Vanhoutte 2006). In terms of CVD, the most important aspect of the endothelium is its ability to maintain vascular tone. Endothelium can synthesize and release several vasoactive products which can mediate the relaxation and constriction of the vessel (Vallance 2001). For example, endothelin-1 and thromboxane-A2 act as vasoconstrictors whereas, endothelium derived relaxing factor (EDRF) (later identified as nitric oxide), prostacyclin and endothelium-derived hyperpolarizing factor (EDHF) act as vasodilators (Vallance 2001). The main regulator of the endothelium mediated vasorelaxation is thought to be EDRF. EDRF causes vasorelaxation by facilitating the stimulation of vessels with intact endothelium by acetylcholine (Furchgott and Zawadzki 1980). EDRF release is stimulated by various agonists and it is a diffusible and unstable substance which acts on vascular smooth muscle cells to cause relaxation. Later studies have confirmed that EDRF is actually NO· (Furchgott and Zawadzki 1980). Nitrovasodilators (exogenous NO· donors) have been in use for about a century to date without understanding their mechanism of action (Pacher et al. 2007). Nitrovasodilators such as nitroglycerine and sodium nitroprusside have been shown to stimulate vascular relaxation by activating soluble guanylate cyclase thus elevating cyclic guanylate monophosphate (cGMP) (Katsuki et al. 1977). Increased levels

of cGMP produced by guanylate cyclase within vascular smooth muscle cells were discovered to allow blood vessels to relax, which in turn increases the blood flow (Ignarro et al. 1986). Although, nitrovasodilators are able to activate the enzyme guanylate cyclase, the endogenous agents responsible for activating guanylate cyclase remained elusive. Later on it was discovered that $NO^{\cdot}$ is able to activate guanylate cyclase (Andrews et al. 2002). $NO^{\cdot}$ acts as the major vasodilator in conduit arteries such as the aorta whereas, EDHF acts as the principle vasodilator in resistant arteries such as the coronary artery (Avogaro et al. 2006).

1.2.5.2(b) Endothelial Dysfunction

Endothelial dysfunction is defined as the inability of the endothelium to function optimally. Endothelial dysfunction is characterized by an increased vascular tone, liability towards vasospasm and irregularities of blood flow. Genetic and traditional risk factors for CVD have been shown to contribute to the impairment of endothelial function (Celermajer et al. 1993; Clarkson et al. 1997; Komatsu et al. 2002). Prolonged and repeated exposure to cardiovascular risk factors can ultimately exhaust the protective effect of the endothelial cell layer. As a consequence, the endothelium becomes dysfunctional (Woywodt et al. 2002). Endothelial function is maintained in a tightly controlled balance. The disruption of this balance leads to the development of endothelial dysfunction. Endothelial dysfunction represents early features of diabetes, hypertension, cardiac failure and atherosclerosis (Feletou and Vanhoutte 2006). Although the characteristic of endothelial dysfunction is the impairment of the endothelium-dependent vasodilation other complications such as inflammation, elevated oxidation of lipoproteins, vascular smooth muscle cell proliferation and migration, extracellular

matrix deposition, platelet activation and thrombus formation are also important in cardiovascular diseases (Escobales and Crespo 2005). Another important factor is that the endothelial dysfunction associated with the above listed diseases is related to the local formation of ROS and RNS in the proximity of the vascular endothelium (Escobales and Crespo 2005; Griendling et al. 2000; Jay et al. 2006; Li and Shah 2004; Linke et al. 2003).

Endothelial dysfunction during HHcy occurs via several mechanisms. Hcy has been shown to have a direct cytotoxic effect on endothelial cells *in vitro* (Harker et al. 1976; Wall et al. 1980). Hcy can undergo auto-oxidation to form Hcy while releasing a $O_2^{\cdot-}$ anion. The $O_2^{\cdot-}$ anion is eventually converted to H_2O_2 and $OH^{\cdot}$ (Loscalzo 1996). H_2O_2 contributes to Hcy-induced endothelial injury which would act as a predisposing factor for the atherogenic process (response to injury) (Starkebaum and Harlan 1986). However, during *in vivo* situations, endothelial injury due to HHcy was not observed. The postmortem examination of the vessels of homocysteinuric patients did not show any physical damage to the endothelium although atherosclerotic lesions were observed (Busse and Fleming 1996; Drexler and Hornig 1999). Therefore, the current research has shifted the focus more towards the functional damage to the endothelium rather than the mechanical damage occurring during HHcy. Functional damage of the endothelium is associated with the reduced bioavailability of $NO^{\cdot}$ during HHcy (Suematsu et al. 2007).

1.2.5.2(c) Clinical Assessment of Endothelial Function

Under physiological conditions, the endothelium is capable of expanding to the increase of blood flow by increasing the $NO^{\cdot}$ production mediated by 'shear stress' (Corretti et al. 2002).

This effect is termed as flow mediated dilatation (FMD). FMD can be measured by artificially increasing blood flow through a selected artery (Corretti et al. 2002). This is a clinical assessment used to measure the endothelial function in humans by using ultrasonic wall tracking of the brachial artery (Corretti et al. 2002). Blood flow through the artery is increased by causing an ischemic period (blockage) proximal to the brachial artery and by releasing the blockage causing a hyperemic condition down stream (Corretti et al. 2002). Measuring the vessel diameter before and after the increase of blood flow gives a value of the endothelium-dependent vasodilatation (Corretti et al. 2002). As FMD is an *in vivo* assessment of endothelial function, the use of an organ bath is an *in vitro* assessment of endothelial function. In an organ bath study segments of aortas are contracted by exposing them to a vasoconstrictant and then exposing them to acetylcholine to determine the degree of relaxation. Assessment of endothelial function in this manner has been in use extensively in cardiovascular research. Several prospective studies have shown that endothelial function is an early predictor of the development of atherosclerosis and CVD (Halcox et al. 2002; Schachinger et al. 2000). Therefore, it is logical to infer that any interventional therapy that could improve endothelial function could have a beneficial effect on cardiovascular diseases.

1.2.5.2(d) Circulating Markers of Endothelial Function

The degree of endothelial function/dysfunction can be assessed to some extent by specific circulating molecular markers. These include the direct products of the endothelium that are released by the activation of endothelial cells including metabolites of $NO^{\cdot}$, inflammatory cytokines, adhesion molecules, von Willebrand factor, regulators of thrombosis and markers of endothelium damage and repair (Deanfield et al. 2007). Most

of these circulating markers are difficult and expensive to measure as routine clinical tests. However they provide valuable information regarding the mechanisms involved and the severity of the dysfunction (Rassaf et al. 2004; Smith et al. 2004). The circulating levels of nitrites, nitrates and nitrosylated proteins reflect the endothelial generation of $NO^{\cdot}$ but may not always represent the endothelium specific $NO^{\cdot}$ production. The values may be confounded due to the formation of adducts from other nitrogen containing species, sources other than the endothelium derived $NO^{\cdot}$ and dietary $NO^{\cdot}$ (Rassaf et al. 2004).

1.2.5.3 Nitric Oxide and Peroxynitrite

$NO^{\cdot}$ is a ubiquitous small molecule that acts as an intracellular messenger in all vertebrates (Pacher et al. 2007). $NO^{\cdot}$ is removed from tissues by the rapid diffusion through tissues into erythrocytes where it is converted into nitrate by oxyhemoglobin (Joshi et al. 2002). This prevents even the highest concentrations of $NO^{\cdot}$ that is reactive with molecular oxygen to form nitrogen dioxide (NO_2). The simultaneous synthesis of $O_2^{\cdot-}$ adjacent to $NO^{\cdot}$ can change the biological activity of $NO^{\cdot}$ by synthesizing $ONOO^{-}$.

$$O_2^{\cdot-} + NO^{\cdot} \rightarrow ONOO^{-}$$

The $O_2^{\cdot-}$ reacts with $NO^{\cdot}$ at an extremely fast rate (Meli et al. 2002). The increase of $NO^{\cdot}$ and $O_2^{\cdot-}$ production by 10 fold would lead to the increase of $ONOO^{-}$ production by 100 fold whereas, an increase of 1000 fold would lead to 1,000,000 fold increment of $ONOO^{-}$ (Liochev and Fridovich 2003; Liu et al. 1998). The half life of $NO^{\cdot}$ *in vivo* is about 1-3 seconds (Feelisch et al. 1994; Moncada et al. 1989; Pacher et al. 2007). When $O_2^{\cdot-}$ and $NO^{\cdot}$ are synthesized within close proximity in a cell, they will combine to form $ONOO^{-}$

by a diffusion limited reaction (Beckman et al. 1994). However, $O_2^{\cdot-}$ and $NO^{\cdot}$ do not need to be produced in the same cell because of the rapid diffusing capacity whereby $NO^{\cdot}$ can move through cell membranes and cells (Pacher et al. 2007). $NO^{\cdot}$ is the only known molecule in the body that reacts fast enough with $O_2^{\cdot-}$, therefore, avoiding the SOD scavenging system (Pacher et al. 2007). The thermodynamics of the reaction makes it unavoidable to combine $NO^{\cdot}$ with $O_2^{\cdot-}$ to form $ONOO^-$. Even though, $NO^{\cdot}$ at concentrations several times higher than the normal physiological concentration is not toxic to tissues, $NO^{\cdot}$ converted into a highly potent reactive $ONOO^-$ is toxic (Pacher et al. 2007). Although $ONOO^-$ is a highly reactive molecule, its reaction with most biological molecules is relatively slow and it can diffuse further into tissues via ion channels (Denicola et al. 1998; Macfadyen et al. 1999). Pathological conditions can greatly increase the production of $ONOO^-$. Even the generation of a moderate level of $ONOO^-$ over a prolong period of time will result in considerable oxidation and subsequent damage to tissues (Virag et al. 2003). This could lead to dysfunction of critical cellular processes, disruption of cellular signaling pathways and induction of cell death through apoptosis and necrosis (Virag et al. 2003). The half life of $ONOO^-$ is short (10-20 ms) but sufficient to cross cell membranes and diffuse through one to two cells (Denicola et al. 1998). This allows significant interactions with critical biomolecules (Pryor and Squadrito 1995). Kinetic studies have shown that $ONOO^-$ oxidizes target molecules through two distinct mechanisms: $ONOO^-$ or its protonated form peroxynitrous acid ($ONOOH$) can cause direct oxidative modification through one or two electron oxidation processes. Only a few chemical groups such as thiols, iron/sulfur centers and zinc fingers can directly react with $ONOO^-$ (Beckman and Koppenol 1996). The second mechanism

involves indirect oxidation via the products of decomposing $ONOO^-$, hydroxyl radicals ($OH^\cdot$) and nitrogen dioxide (NO_2), which is less common in *in vivo* situations (Radi 1998). Nitrotyrosine is extremely useful for measuring the formation of $ONOO^-$ and considered as a marker of 'nitrative' stress. By proteomic analysis it has been shown that the nitration process by $ONOO^-$ is a highly selective process limited to specific tyrosine residues on a small number of proteins (Aulak et al. 2001; Gow et al. 2004; Kanski et al. 2005). Nitration of specific tyrosine residues in protein is facilitated by the exposure of the aromatic ring to the surface of the protein, the location of the tyrosine on a loop structure, its association with a neighboring negative charge and the absence of proximal cysteines (Souza et al. 1999). Tyrosine nitration is favored in a hydrophobic environment which allows $ONOO^-$ to pass through the cell membranes. Protein tyrosine nitration is a covalent protein modification which adds a nitro group adjacent to the $OH^\cdot$ group on the aromatic ring of tyrosine residues (Gow et al. 2004). Tyrosine nitration affects protein structure and function resulting in the change of catalytic activity of enzymes, alteration of cytoskeleton organization, impairment of cell signaling and formation of antigenic epitopes (Schopfer et al. 2003). The first reaction of tyrosine nitration is the removal of a hydrogen atom from tyrosine to form a tyrosyl radical that combines with NO_2 to produce 3-nitrotyrosine, which competes with a secondary reaction by combining with another tyrosyl radical to form dityrosine (Beckman 1996; Beckman and Koppenol 1996; Ischiropoulos 2003; Radi 2004).

1.2.5.4 Nitric Oxide Synthase

$NO^\cdot$ is synthesized by the conversion of amino acid L-arginine to L-citrulline by nitric oxide synthase (NOS) (Palmer et al. 1988). There are three isozymes of NOS, namely,

endothelial nitric oxide synthase (eNOS), inducible nitric oxide synthase (iNOS) and neuronal nitric oxide synthase (nNOS). They are similar in their structure but regulated by different mechanisms. NOS contains 5 domains; iron protoporphyrin, FAD, flavin mononucleotide (FMN) and tetrahydrobiopterin for the binding of cofactor groups (Stuehr 1997). All of the three isozymes activation depends on the binding of calmodulin, the calcium (Ca^{2+}) binding regulatory protein. For the activation of eNOS and iNOS, higher levels of Ca^{2+} than the resting Ca^{2+} ion levels are required for the binding of calmodulin whereas, to activate iNOS Ca^{2+} ions at resting (basal) concentration are adequate to bind calmodulin (Baek et al. 1993; Stuehr et al. 1991). Therefore, iNOS activity is independent of intracellular Ca^{2+} concentration (Baek et al. 1993; Stuehr et al. 1991). The intracellular activities of eNOS and nNOS are closely regulated by changes in Ca^{2+}, whereas, the iNOS in phagocytic cells such as macrophages and neutrophils is not regulated by intracellular Ca^{2+} concentration (Nathan and Xie 1994).

CHAPTER 2

MATERIALS AND METHODS

2.1 *In Vivo* Hyperhomocysteinemic Model

Male Sprague-Dawley rats weighing 200-250 g were obtained from the Charles River Laboratories (Wilmington, MA, USA) and quarantined in the St. Boniface Hospital Research Center animal Care Facility for a period of 1 week. They were fed with Lab Diet Rodent 5001 containing different levels of methionine for 4 weeks. The control diet consisted of regular diet with a basal methionine level of 0.43% (Au-Yeung et al. 2004; Wang et al. 2002). The high methionine diet consisted of regular diet with 1.7% (weight/weight) methionine (Sigma-Aldrich, St. Louise, MO, USA). A group of the high methionine fed group was injected with 4 mg/kg apocynin (Calbiochem, San Diego, CA, USA) intraperitoneally once daily for 7 days prior to euthanasia. Each group consisted of 12 rats. All animal handling procedures were performed in accordance with the Guide to the Care and Use of Experimental Animals published by Canadian Council of Animal Care (1993) and the experiment was approved by the University of Manitoba Protocol Management and Review Committee.

2.2 Sample Collection

Animals were injected with 50 mg/kg sodium pentabarbitone (Sigma-Aldrich) intraperitoneally. After complete anesthesia whole blood samples were collected for serum homocysteine analysis. Thoracic aortas were isolated and immediately frozen at -80°C.

2.3 Measurement of Serum Homocysteine Concentrations

The whole blood samples were collected and serum was separated by centrifuging at 3,000g for 20 minute at 4°C. 250 µl of the supernatant was collected and kept at -20°C until the analysis. The total Hcy concentration in the serum was measured with IMx Hcy

assay based on the fluorescence polarization immunoassay technology (Abbots Diagnostics, Abbot Park, IL, USA) (Au-Yeung et al. 2004; Wang et al. 2002).

2.4 Detection of Superoxide Anions in Aorta

Thoracic aorta was isolated and the aortic segments were immediately frozen at -80°C and embedded in Tissue-Tek optimum cutting temperature compound (O.C.T., Sakura, Finetek, Torrance, CA, USA). Sequential cross sections (5 μm) were prepared and the oxidative fluorescent dye dihydroethidium (DHE) (Sigma-Aldrich) was used to detect $O_2^{\cdot-}$ anions in the aorta. An increase in the fluorescence intensity under a confocal microscope (Bio-Rad MRC-1024UV) reflected an increase in $O_2^{\cdot-}$ anions in the aorta (Au-Yeung et al. 2004).

2.5 Determination of NADPH Oxidase Activity in Aorta

The NADPH oxidase activity was measured by lucigenin enhanced chemiluminescence assay that was described by Chen et al. (Chen X et al. 2001). Frozen aortas were sectioned to 5 mm rings, trimmed off the excess fat carefully and equilibrated in HEPES-Krebs Henseilete buffer (HEPES-KH, 25 mmol/L $NaHCO_3$, 99.01 mmol/L NaCl, 11.1 mmol/L Dextrose, 4.69 mmol/L KCl, 1.2 mmol/L $MgSO_47H_2O$, 1.03 mmol/L K_2HPO_4, 1.87 mmol/L $CaCl_2$ and 20 mmol/L HEPES, pH 7.4) at 37°C and 5% CO_2 for 15 minute. The aortic rings were carefully transferred into a luminometer tube containing 450 μmol/L HEPES-KH buffer and 5 μmol/L lucigenin (Sigma-Aldrich) and placed in the luminometer (Lumet LB9507, Berthold Technologies GmbH & Co. KG, Bad Wilbad, Germany). The luminometer was set up to add 100 μmol/L of NADPH, the substrate for NADPH oxidase to the reaction tube. The luminometer measured the NADPH oxidase activity for 3 minutes for every 15 seconds. In principle the reaction of lucigenin with

$O_2^{\cdot-}$ anion leads to the formation of lucigenin dioxetane that decomposes to produce two molecules of N-methylacridone. One of these two N-methylacridone molecules is in an electronically excited state and emits a photon. The photon emission that reflects the amount of $O_2^{\cdot-}$ anions in the sample can be detected using the luminometer. The standard curve was prepared by using xanthine (100 µmol/L) (Sigma-Aldrich) and known serial concentrations of xanthine oxidase (Sigma-Aldrich). 1:40 or 1:50 diluted xanthine oxidase, 0 µl, 2 µl, 4 µl and 8 µl volumes were add to the HEPES-KH buffer 450 µl, 448 µl, 446 µl, and 442 µl volumes correspondently. 1 unit of xanthine converts 1.0 µmol of xanthine to uric acid per minute i.e. 1 µmol $O_2^{\cdot-}$ per minutes. The calculation of NADPH oxidase activity was based on the amount of $O_2^{\cdot-}$ anions produced during the reaction. Standard curve consists of log relative light unit (RLU) at the y axis and $O_2^{\cdot-}$ production at the x axis.

2.6 Determination of Protein Concentration

For samples with protein concentrations lower than 10 µg/ml, the protein concentration was measured by Bio-Rad Protein Assay (Bio-Rad Lab, Hercules, CA, USA). The assay was based on the Bradford method. The dye reagent concentrate was diluted (1:5) with distilled deionized water (DH_2O). A standard solution of 1 mg/ml BSA (Sigma-Aldrich) was made by diluting BSA in DH_2O. Different concentrations - 0 µl, 2 µl, 4 µl, 6 µl and 8 µl volumes of standard BSA and 1000 µl, 998 µl, 996 µl, 994 µl, 992 µl volumes of diluted dye reagent were mixed and incubated for 5 minutes and the absorbance was measured at 595 nm by a Beckman Spectrophotometer (Beckman coulter, Fullerton, CA, USA) to make the standard curve. 2 µl volume of the sample was mixed and incubated with 998 µl volume of the dye reagent to measure the protein levels of the samples.

2.7 Western Immunoblot Analysis in Aorta

Protein levels of eNOS and NADPH oxidase subunits were determined by western inmmunoblot analysis (Siow et al. 2006). A portion of the aorta (2 cm) was homogenized in a buffer containing 10 mmol/L Tris-hydrochloric acid (Tris-HCl, pH 7.5), 5 mmol/L ethylenediaminetetraacetic acid (EDTA), 10 mmol/L ethylene glycol tetraacetic acid (EGTA), 0.2 mmol/L phenylmethanesulphonylfluoride (PMSF), and 20 μmol/L leupeptin. Homogenate was sonicated at 4 mW power for 5 seconds, and centrifuged at 3000 g for 10 minutes. The protein concentration of the supernatant was measured using the Bradford method. Proteins were equalized, so that 1 μl of a sample contains approximately 2-3 μg of protein. A sample buffer containing 5x sodium dodecylsulfate (SDS, 250 mmol/L Tris-base, pH 6.8, 25% glycerol, 10% SDS, 0.02% bromophenol blue and 5% β-mercaptoethanol) was added to the samples and heated at 95˚C for 5 minutes to denature the proteins. Proteins (40-60 μg) in the samples were separated by their molecular weight by running them through a gel containing 8-15% polyacrylamide gel (30% acrylamide bis solution, Tris and SDS) at 150 V constant volts for 1 hour or until the gel front reaches the bottom of the gel using a Bio-rad mini gel apparatus. The proteins in the gels were transferred to 0.45 μm nitrocellulose membranes (Bio-Rad) at 300 mA constant current for 30 minutes. The membranes were blocked for non specific protein with 2% BSA in Tris-buffered solution with 0.1% Tween-20 (TBST) for 1 hour at room temperature. After washing the membranes with TBST for 3 times 10 minutes each, they were incubated (gently shaking) with the primary antibodies diluted 1:1000 in 0.2% BSA in TBST at 4˚C for overnight. The membranes were washed with TBST for 3 times for 10 minutes each. Then the membranes were probed with horse raddish peroxidase

linked secondary antibodies (Zymed, Laboratories Inc., San Francisco, CA, USA) for 1 hour at room temperature (gently shaking). The membranes were washed twice with TBST and once with TBS for 10 minutes each. The bands corresponding to the target proteins were visualized with enhanced chemiluminescene reagent (ECL, Amersham, Piscataway, NJ, USA) and exposed to Kodak X-Omat Blue XB-1 films. The results were analyzed by Bio-Rad quantity one image analysis soft ware (version 4.2.1.)

2.8 Determination of Messenger Ribonucleic Acid Expression in Aorta

Messenger ribonucleic acid (mRNA) expression of eNOS and NADPH oxidase subunits in the aorta and cultured vascular cells were determined by semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR) .

2.8.1 Isolation of Total Ribonucleic Acids

A portion of frozen aorta (1 cm) was homogenized in TRIzol reagent (Invitrogen, Carlesbad, CA, USA). A volume of 200 µl of chloroform (Fisher scientific, Fair Lawn, NJ, USA) was added to the homogenate and vigorously shaken by the hand for 15 seconds and incubated at room temperature for 2 minutes. The homogenate was centrifuged at 12,000 g at 4°C for 15 minutes. The top aqueous layer was collected (400 µl-500 µl) and mixed with 500 µl of isopropyl alcohol (Fisher scientific), vigorously shaken by the hand, left at room temperature for 10 minutes and centrifuged at 12,000 g for 10 minutes. The resulting supernatant was discarded and the RNA pellet was washed with 1 ml of 75% ethanol and centrifuged at 12,000 g for another 10 minutes. The RNA pellet was dried for 10 minutes and dissolved with 12-20 µl of diethylpyrocarbonate (Sigma-Aldrich) water (DEPC H_2O) and incubated at 56°C for 10 minutes. RNA concentration was measured adding 2 µl of RNA into 98 µl of DEPC H_2O and read at

260 nm and 280 nm by a spectrophotometer (Beckman coulter). RNA was equalized with DEPC H_2O for each sample therefore the final RNA concentration was 5 µg in 10 µl.

2.8.2 Reverse Transcriptase Reaction

Total RNA (5 µg) was converted into complementary DNA (cDNA). RNA samples were mixed with 10 µl of RT mixture containing 1 x 1st strand buffer, 0.5 mmol/L 2'-deoxynucleoside 5'-triphosphate (dNTP), 10 mmol/L DL-dithiothreitol (DTT), 10 ng/µl oligo dT, 1 unit/µl RNAase inhibitor (RNasin) and 1 unit/µl reverse transcriptase (M-MLV-RT). The samples were left at room temperature for 10 minutes and incubated at 37°C for 1 hour. The reverse transcriptase activity was inhibited by heating the samples at 95°C for 2 minutes (Sung et al. 2002).

2.8.3 Polymerase Chain Reaction

10 µl of cDNA product from the RT reaction was mixed with 40 µl of PCR reaction mixture containing 1x PCR buffer (10 mmol/L Tris-HCl, 50 mmol/L KCl and 1.5 mmol/L $MgCl_2$), 0.2 mmol/L dNTP, 0.4 mmol/L forward primer, 0.4 mmol/L reverse primer, 2 units of *Taq*-DNA polymerase and sterilized distilled water. And the cDNA was amplified by PCR (Bio-Rad) (Sung et al. 2002).

The primers used for $p22^{phox}$ in the PCR reactions were (forward) 5'-GACGCTTCACGCAGTGGTACT-3' and (reverse) 5'-CACGACCTCATCTGTCA CTGG-3'. The primers used for $p47^{phox}$ were (forward) 5'-TCACCGAG ATCT ACGAGTTC-3' and (reverse) 5'-TCCCATGAGGCTGTTGAAGT-3'. The primers used for $p67^{phox}$ were (forward) 5'-CGAGGGAACCAGCTGATAGA -3' and (reverse) 5'-CATGGGAACACTGAGCTTCA -3'. The primers for $gp91^{phox}$ were (forward) 5'-AGCTAGAGTGGCACCCATTC-3' and (reverse) 5'-TCCCAGTTGGGCCGTCCATA-

3'. The primers for NOX1 were (forward) 5'- CCCGCAACTGTTCATACTC -3' and (reverse) 5'- CATTGTCCCACATTGGTCTC -3'. The primers for NOX4 were (forward) 5'- CTGCATCTGTCCTGAACCTCAAC-3' and (reverse) 5'-AGCCAGGAGGGTG AGTGTCTAA-3'. The primers used for eNOS were ((Forward) 5'–CTA GAG GCT CT CAGCCAGGA. (Reverse) 3'-GGCTGCAGTCCTTTGATCTC. All primers were syn thesized by Invitrogen.

The numbers of cycles of PCR amplification were 30 cycles for all the subunits (95°C for 55 seconds, 58°C for 40 seconds and 72°C for 90 seconds). An additional 7 minutes extension was carried out at 72°C). The PCR products were separated by running through a gel containing 1.5% agarose (Invitrogen) and 0.5 μg/ml ethidium bromide (Sigma-Aldrich), by electrophoresis. The gel was visualized under ultraviolet light with a gel documentation system (Bio-Rad Gel Doc 1000). Rat glyceraldehydes-3 phosphate dehydrogenase (GAPDH) was used as an internal control to verify the equal loading of PCR product for each experiment. The values were expressed as percentages of ratio of the mean intensity of the target PCR band to GAPDH band.

2.9 *In Vitro* Models: Culture of Vascular Cells

Human Umbilical Vein Endothelial Cells (HUVEC) were purchased from the American Type Culture Collection (ATCC, Manassas VA, USA). Cells were cultured in F-12K nutrient medium (Hyclone, Logan, UT, USA) containing 0.5% of endothelial cell growth supplement (ECGS, BD Biosciences, San Jose, CA, USA) and 20% fetal bovine serum (FBS, Hyclone) in 75mm^2 flasks (Falcon) containing 10 ml of medium. The medium was changed 24 hours after the sub culture and then after every 2 days until the cells were

80% confluent. HUVEC between passages 2-8 were used for the experiments according to the supplier's instruction.

Vascular Smooth Muscle Cells (VSMC) were purchased from the American Type Culture Collection (ATCC). Cells were cultured in F-12K nutrient medium (Hyclone) containing 0.25% ECGS (BD Biosciences), insulin transferrin sodium selenite solution (ITS, 10 µg/ml of insulin, 10 µg/ml transferrin, 10 ng/ml sodium selenite) and 10% FBS (Hyclone). Medium was changed 24 hour after the sub culture and then after every 2 days until the cells were 80% confluent. VSMC between passages 18-22 were used for experiments according to the supplier's instruction.

2.10 Determination of Intracellular Superoxide Anion Levels in Vascular Cells

Both HUVEC and VSMC were grown until 80% confluent. The medium was removed and the cell monolayer was washed with colorless Hanks modified salt buffer (Hanks, Hyclone). Cell monolayer was detached with 0.05% trypsin-EDTA (Hyclone). Cells were centrifuged at 130 g for 8 minutes at room temperature. Cells were re-suspended in culture medium and counted using a hemocytometer. Cells were re-seeded in 6-well culture plates so that the final cell number was 2×10^5/ ml. The medium was changed into serum free medium before 18 hours had elapsed to make the cells quiescent. After 24 hours of serum deprivation, 1 ml of fresh serum free medium was added to each well and the treatment commenced. Cells were pre-incubated with 300 µmol/L apocynin. For HUVEC the apocynin pre-incubation period was 30 minutes and for VSMC it was 2 hours (Optimal pre-incubation time was determined empirically after conducting a pre-incubation time course). Then cells were treated with 100 µmol/L DL-Hcy for 30 minutes (Au-Yeung et al. 2004; Siow et al. 2006). At the end of the incubation time the cells were

washed with Hanks buffer 3 times. 0.1% Nitrobluetetrazolium (NBT) solution was prepared by dissolving NBT in Krebbs Henseleite buffer (KH, 120 mmol/L NaCl, 25 mmol/L $NaHCO_3$, 5.5 mmol/L Dextrose, 4.76 mmol/L KCl, 1.2 mmol/L $MgSO_47H_2O$, 1.2 mmol/L KH_2PO_4, and 1.27 mmol/L $CaCl_2$ $2H_2O$) (Au-Yeung et al. 2004; Wang et al. 2000). 1 ml from 0.1% NBT solution was added to each well (Au-Yeung et al. 2004; Wang et al. 2000) and incubated for 45 minutes. After checking the development of intracellular blue color under a light microscope (10x power), the cells were washed with PBS buffer at room temperature 3 times and dried completely. The cells were lysed with 1 ml of 80 mmol/L phosphate lysis buffer (5% SDS, 0.45% gelatin, 0.2 mol/L Na_2HPO_4 and 0.2 mol/L NaH_2PO_4 at pH 7.8) to release the intracellular formazan. Cell lysate was collected and centrifuged at 13,000 g for 3 minutes at room temperature. The supernatant was collected and the absorbance of the blue color formazan was read at wave lengths of 450 nm (background) and 540 nm (signal). Calculations were done by deducting the readings at 450 nm from the readings at 540 nm (Au-Yeung et al. 2004; Wang et al. 2000). The mean values were expressed as percentages of control. The initial standard curve for formazan was formed by using a 1 mg/ml NBT formazan solution.

2.11 Western Immunoblot Analysis in Vascular Cells

Western immunoblot analysis was performed for the determination of protein expression in vascular cells (Siow et al. 2006). Both HUVEC and VSMC were grown until 80% confluent. Cells were collected as previously mentioned and seeded 2 x 10^6/ml in 100 mm culture plates. Medium was changed into serum free before 18 hours had elapsed. After 24 hours in serum free medium the cells were treated with 100 μmol/L DL-Hcy in the culture medium for 6 hours for HUVEC and 4 hours for VSMC. At the end of the

treatment time the cell monolayer was washed 3 times with PBS at 4°C and dried completely. 150 µl of western lysis buffer (10 mmol/L Tris-HCl, 5 mmol/L EDTA, 10 mmol/L EGTA, 0.2 mmol/L PMSF and 20 µmol/L leupeptin at pH 7.5) was added to each plate and the cells were scraped and collected. Cell lysates were sonicated at 4 mW power for 5 seconds and centrifuged at 3,000g for 10 minutes. The supernatant was collected and the protein concentration was measured using the Bradford method. Samples were equalized for protein that each sample contained 2-3 µg of protein per 1 µl. Rest of the procedure was done as previously mentioned (Siow et al. 2006).

2.12 Sample Preparation with Vascular Cells for Total RNA Isolation

Both HUVEC and VSMC were grown until 80% confluent. Cells were collected as previously mentioned and seeded 2 x 10^5/ml in 60 mm culture plate. The medium was changed into serum free medium before 18 hours had elapsed to make the cells quiescent. After 24 hours of serum deprivation, 2 ml of fresh serum free medium was added to each plate and the treatment commenced. Cells were treated with 100 µmol/L DL-Hcy in the culture medium for 6 hours for HUVEC and for 4 hours for VSMC at 37°C and 5% CO_2. At the end of the treatment time the cell monolayer was washed 3 times with PBS at 4°C and dried completely. 1 ml of TRIzole reagent was added per plate and the cells were lysed by repetitive pipetting on ice. Cell lysates in TRIzole were immediately used for total RNA isolation or stored at -80°C. RNA isolation was performed as previously mentioned (Sung et al. 2002).

2.13 $p22^{phox}$ Short Interrupted RNA (siRNA) Transfection in vascular cells

Post transcriptional gene knockdown method was utilized in vascular cells by transfecting the cells with $p22^{phox}$ siRNA duplex (Santa Cruz Biotechnology, Santa Cruz,

CA, USA) to knockdown $p22^{phox}$ subunit accoriding to the method described by Siow et al. (Siow et al. 2006). HUVEC or VSMC were seeded 2×10^5/ml in 6-well plates and transfected with $p22^{phox}$ siRNA duplex (sc-29422) according to the manufacturer's instructions. For a negative control, cells were transfected with a control siRNA duplex (sc-36869) consisting of a scrambled sequence that did not knock down cellular RNA (Santa Cruz). At 48 hours after transfection, cells were washed three times with F-12K medium. Cells were incubated in the presence or absence of 100 μmol DL-Hcy. The intracellular $O_2^{\cdot-}$ level was measured by NBT reduction assay as described previously. The mRNA level of $p22^{phox}$ in transfected cells were determined by RT-PCR analysis. The primers used for human $p22^{phox}$ were (forward) 5'-GTTTGTGTGCCTGCTGGAGT-3' and (reverse) 5'-TGGGCGGCTGCTTGATGGT-3' (Invitrogen).

2.14 Assessment of Endothelial Function

The aortic ring segments (5 mm) were prepared from rat thoracic aortas. The endothelium-dependent relaxation function of the aortas was examined by measuring the response to acetylcholine (Sigma-Aldrich) (Dupasquier et al. 2006; Wang et al. 2002). Precautions were taken to avoid excessive stretch of the aorta or damage of the endothelium during the preparation of the rings (Dupasquier et al. 2006; Wang et al. 2002). In brief, aortic ring segments were placed in an organ bath chamber containing Krebs-Henseleit buffer. A standard tension (1.5 g) was applied to the aortic rings (Wang et al. 2002). After the aortic rings were equilibrated for 90 minutes in the organ bath, the rings were contracted with 10^{-6} mol/L phenylephrine (Sigma-Aldrich). Endothelium-dependent vessel relaxation was determined as the response of the rings to acetylcholine (10^{-10} to 10^{-6} mol/L).

2.15 Detection of Peroxynitrite in Aorta

$ONOO^-$ levels were detected in aortas by measuring its biomarker-nitrotyrosine protein adduct by immunohistochemical staining (Zhang et al. 2004). Frozen aortas were cut into 5 mm rings and freeze embedded in Tissue-Tek O. C. T compound using a 1.5cm x 1.5cm square mold. Sequential cross sections of 5 μm were prepared at -20°C using a cryotome (Shandon cryostat, Life Sciences, Cheshire, WA, England). Aortic Sections were immediately fixed on Vectabond treated glass slides (VWR International, West Chester, PA, USA) with 10% phosphate buffered formalin for 1 hour. The slides were washed with phosphate buffered saline (PBS) 3 times after every step. The slides were immersed in 100% methanol at 4°C for 6 minutes to permeabilize the cell membranes. Non specific protein-antibody binding was prevented by incubating the sections in 2% bovine serum albumin (BSA, Sigma-Aldrich) for 1 hour. The aortic sections were incubated with mouse anti-nitrotyrosine primary antibodies (1:100, Zymed) at 4°C for overnight. Negative control was incubated with Mouse IgG (Santa Cruz). The sections were treated with 0.3% H_2O_2 (Fisher scientific) for 20 minutes at room temperature to quench the endogenous peroxidases. Sections were incubated with secondary antibodies of biotin- conjugated anti-mouse immunoglobulins (1:200, Dako Cytomation, Carpinteria, CA, USA) for 1 hour followed by peroxidase conjugated streptavidin (1:250, Zymed) for 50 min. sections were then washed twice with PBS followed by once with Tris-buffered solution (TBS, 0.05mol/L, pH 7.8). Sections were then treated with 3, 3-diaminobenzidine (DAB, Sigma-Aldrich)-H_2O_2 colorimetric substrate solution for approximately 2 minutes. The sections were washed first with running tap water for 2 minutes and with distilled water for another 2 minutes. The nuclei of the cells of the sections were stained by using Mayer's hematoxylin (Sigma - Aldrich) for 6 minutes.

Then the sections were washed with running tap water for 5 minutes and with distilled water for 1 minute. The sections were mounted using 80% glycerol. The peroxidase attached to the secondary antibodies catalyzed the H_2O_2 mediated oxidation of DAB yielding the brown color (Zhang et al. 2004). The images of aortic sections (5 per section) were captured by an Axioskop2 MOT microscope (Carl Zeiss Micro imaging, Thornwood, NY, USA) with an Axiocam camera and analyzed using Photoshop 6.0 (Adobe, San Jose, CA, USA) as follows (Lehr et al. 1999): Images were converted to grayscale mode and inverted (inverted image is the negative of the original image). By using the magic wand tool all the areas except the nuclei and elastic laminas were selected for analysis. The histogram of the image showed the mean luminosity of the selected area. Increased luminosity indicated the increased nitrotyrosine levels in an inverted image.

2.16 Determination of Nitric Oxide Metabolites in HUVEC

The measurement of NO· level in HUVEC was determined by measuring the metabolites of NO·, nitrites and nitrates (Tarpey et al. 2004). HUVEC were grown until 80% confluent and seeded for the experiment in 6-well plates (2×10^5 per well). Medium was changed into serum free F-12K before 18 hours had elapsed. After 24 hours in serum free medium the cells were not treated (control), treated with 100 μmol/L DL-Hcy or pre-treated with 300 μmol/L apocynin for 30 minutes prior to 100 μmol/L DL-Hcy treatment in the culture medium for 6 hours. After 6 hours Hcy treatment, 1 ml of the medium was collected into microcentrifuge tubes and the amount of nitrite and nitrate was determined by the Griess reaction method based on the azo coupling reaction (Tarpey et al. 2004). The sample was first incubated for 25 minutes at 37°C with 10 μl volume of a reaction

mixture containing 2 units of nitrate reductase/500 µl MOP buffer (50 mmol/L MOPS, 0.01 mol/L EDTA, 50% glycerol, 1 mg/ml BSA at pH 7.0), 1 mmol/L FAD and 10 mmol/L NADPH and H_20. This reaction reduces nitrates to nitrites. Meanwhile standard solutions were prepared with Sodium nitrate ($NaNO_2$) from stock solutions of 10 µmol/L and 100 µmol/L. The unreacted NADPH was oxidized by incubating with 10 µl volume of reaction mixture containing 14.5 units of lactate dehydrogenase, 100 mmol/L sodium pyruvate and DH_2O for 10 min at 37°C. After the reaction the tubes were immediately cooled on ice (at 4°C). The azo coupling reaction was completed by adding 12.5 mmol/L sulfanilamide in 6 N HCl and 12.5 mmol/L *N*-(1-naphthyl) ethylenediamine to the samples and the standards. The diazoamino benzene in the reaction mixture was measured by a spectrophotometer at an absorbance of 520 nm. $NaNO_2$ served as the internal standard.

2.17 Statistical Analysis

Results were analyzed by unpaired two-tailed Student's *t*-test for single comparison or using one-way analysis of variance (ANOVA) followed by a Student's Newman-Keuls Multiple Comparison Test for multiple comparisons (GraphPad Prism 3.02 32 Bit Executable) (Almadori et al. 2005; Dixon et al. 2002). Data were presented as the means ± SEM. *P* values less than 0.05 were considered significant.

2.18 Chemicals Used

Chemical/Reagent	**Company**
15ml conical tubes	Falcon®

30% acrylamide bis soulution	Bio Rad Laboratories
75mm^2 culture flasks	BD Biosciences
Acetylcholine	Sigma-Aldrich
Agarose	Invitrogen
Apocynin	Calbiochem
Biotin- conjugated anti- mouse immunoglobulins	Dako
Bromophenol blue	Sigma- Aldrich
BSA	Sigma- Aldrich
$CaCl_2$ $2H_2O$	Sigma- Aldrich
Chloroform	Fisher scientific
DL-HCY	Sigma-Aldrich
DAB	Sigma - Aldrich
DEPC	Sigma - Aldrich
Dextrose	Fisher scientific
DHE	Sigma-Aldrich
dNTP	Invitrogen
ECGS	BD Biosciences
ECL	Amersham
EDTA	Sigma - Aldrich
EGTA	Sigma - Aldrich
Ethidium bromide	Sigma - Aldrich
F-12K nutrient medium	Hyclone

FAD	Sigma - Aldrich
FBS	Hyclone
Gelatin	Sigma - Aldrich
Glycerol	Fisher scientific
H_2O_2	Fisher scientific
Hanks modified phosphate buffer	Hyclone
HCl	EMD
HEPES	Fisher scientific
Horseradish peroxidase linked secondary antibodies	Zymed
HUVEC	ATCC
Isopropyl alcohol	Fisher scientific
ITS	Sigma - Aldrich
KCl	Sigma - Aldrich
KH_2PO_4	Sigma - Aldrich
Lactate dehydrogenase	Sigma - Aldrich
Leupeptin	Fisher scientific
Lucigenin	Sigma- Aldrich
M – MLV-R	Invitrogen
Mayer's hematoxylin	Sigma – Aldrich
Methanol, absolute	Fisher scientific
Methionine	Sigma - Aldrich
$MgCl_2$	Sigma - Aldrich

$MgSO_47H_2O$	Sigma - Aldrich
MOPS	Fisher scientific
Mouse IgG	Santa Cruz Biotechnology
N-(1-naphthyl) ethylenediamine	Sigma - Aldrich
Na_2HPO_4	Sigma - Aldrich
NaCl	Sigma - Aldrich
NADPH	Sigma - Aldrich
NaH_2PO_4	Sigma - Aldrich
$NaHCO_3$	Sigma - Aldrich
$NaNO_2$	Fisher scientific
NBT	Alfa Aeser
Nitrate reductase	Fisher scientific
Nitrocellulose membranes	Bio Rad
O.C.T	Tissue-Tek Sakura
Oligo dT	Invitrogen
$p22^{phox}$ siRNA duplex	Santa Cruz
Peroxidase conjugated streptavidin	Zymed
Phenylephrine	Sigma-Aldrich
PMSF	USB
$Gp91^{phox}$ primary antibodies	Invitrogen
NOX4 primary antibodies	Invitrogen
NOX1 primari antibodies	Invitrogen

$P22^{phox}$ primary antibodies	Invitrogen
$P47^{phox}$ primary antibodies	Invitrogen
$P67^{phox}$ primary antibodies	Invitrogen
Primers	Invitrogen
RNasin	Invitrogen
SDS	Fisher scientific
Sodium pentabarbitone	Sigma-Aldrich
Sodium pyruvate	Fisher scientific
Sodium selinite solution	Sigma-Aldrich
Sulfanilamide	Sigma-Aldrich
Taq – DNA polymerase	New England bio lab
Tris	Sigma- Aldrich
TRIzol reagent	Invitrogen
Trypsin	Hyclone
Tween – 20	Fisher scientific
VSMC	ATCC
X – Omat Blue XB- 1 films	Kodak
Xanthine	Sigma- Aldrich
Xanthine oxidase	Sigma- Aldrich
β – mercaptoethanol	Fisher scientific

CHAPTER 3

RESULTS

3.1 Effect of Hyperhomocysteinemia on Reactive Oxygen Species Production

3.1.1 Serum Homocysteine and Aortic Superoxide Levels

HHcy was induced in rats by feeding a high methionine diet for 4 weeks. Serum samples were collected from all the groups including the control group, the 1.7% methionine fed group (HHcy) and the 1.7% methionine fed and apocynin treated group (HHcy + Apocynin). Serum Hcy concentrations of the above groups were measured by the IMx spectrophotometer. A significant increase in serum Hcy concentrations was detected in HHcy rats compared with the control rats (29.12±2.708 μmol/L, versus 5.84±0.7195 μmol/L) (Figure 3.1). Apocynin treatment did not change the serum Hcy level in the HHcy + Apocynin group (29.40±4.471 μmol /L) (Figure 3.1).

Aortas were isolated from the control, HHcy and HHcy + Apocynin injected groups of rats and the levels of $O_2^{\cdot-}$ in the aortas were measured by a method using DHE fluorescence staining. The cross sections of aortas isolated from HHcy rats displayed a marked increase in DHE fluorescence intensity in the endothelium and sub endothelial tissue, reflecting an increase in $O_2^{\cdot-}$ in the intima as well as in the media and adventitia (Figure 3.2.a). One group of HHcy rats were administered with apocynin, an inhibitor for NADPH oxidase. Treatment of HHcy rats with apocynin reversed the HHcy induced elevation of $O_2^{\cdot-}$ levels (Figure 3.2.a). There was a significant elevation of the $O_2^{\cdot-}$ in the HHcy rats compared with control rats (Figure 3.2.b). The HHcy + Apocynin groups showed a significant reduction in the $O_2^{\cdot-}$ compared with the HHcy rats (Figure 3.2b.).

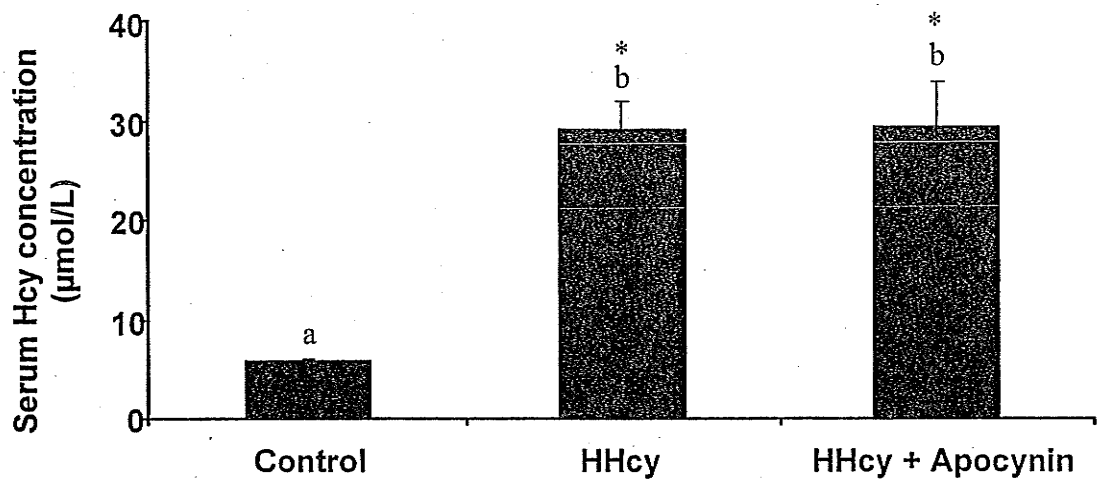


Figure 3. 1. Serum homocysteine concentrations in serum samples collected from the control, HHcy and HHcy + Apocynin groups

Hcy concentrations were measured by IMx spectro photometer. Values without a common letter superscript are significantly different ($P<0.05$). *P<0.05 compared with the value of the control group.

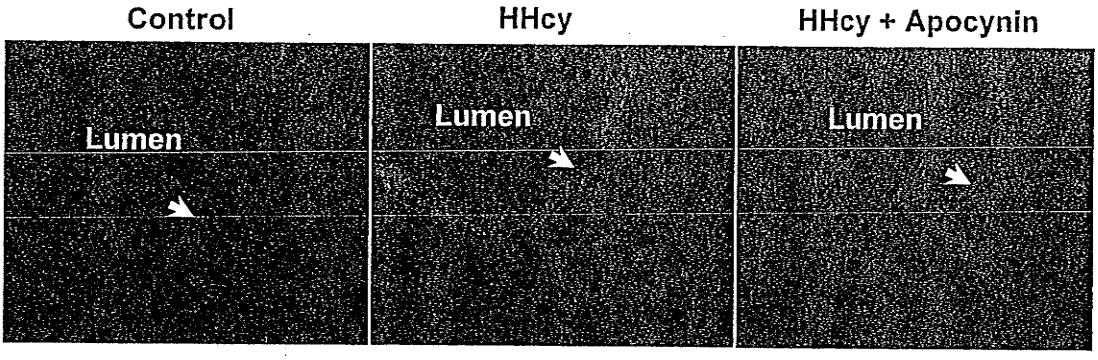


Figure 3. 2a. ***In situ*** **levels of superoxide anions in the thoracic aortas isolated from the control, HHcy and HHcy + Apocynin groups**

Cross cryosections of aortas were prepared. $O_2^{\cdot -}$ were detected in aortas by incubating the cross sections with a fluorescent dye dihydroethidium (DHE). An increase in the fluorescence intensity (red fluorescence) under a confocal microscopy reflected an increase in $O_2^{\cdot -}$ levels in the vessel wall. Representative photos were taken from 5 separate experiments

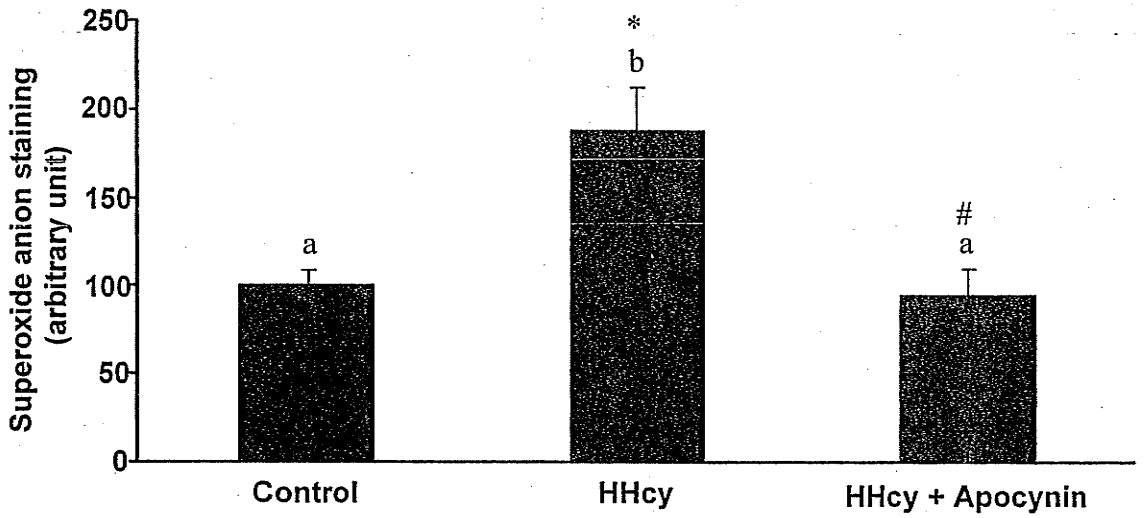


Figure 3. 2b. ***In situ*** **levels of superoxide anions in the aorta**
The intensity of red fluorescence of the DHE stained cross sections of aorta was quantified using Photoshop 6.0. Results were expressed as mean ± SEM. Values without a common letter superscript are significantly different ($P<0.05$). *P<0.05 compared with the value of the control group. #P<0.05 compared with the value of the HHcy group.

3.1.2 NADPH Oxidase Activity and Subunit Expression

The NADPH oxidase activity was measured in the aortas. The NADPH oxidase activity in the aortas isolated from HHcy rats was significantly higher than that of the control rats (Figure 3.3). Administration of apocynin effectively reduced NADPH oxidase activity in HHcy rats (Figure 3.3). Next, we investigated whether activation of NADPH oxidase in the aorta was due to changes in protein and mRNA levels of individual subunits of the enzyme. There was a significant elevation in the protein level of $p22^{phox}$ in the aortas of HHcy rats (Figure 3.4). Consistent with this change, the mRNA level of $p22^{phox}$ was markedly elevated in the same aortas (Figure 3.5). However, the protein and mRNA levels of other NADPH oxidase subunits were not significantly changed in the aortas of HHcy rats as compared to that of control rats (Figure 3.4 and 3.5). These results suggested that increased expression of $p22^{phox}$ subunit might contribute to NADPH oxidase activation in the aorta during HHcy.

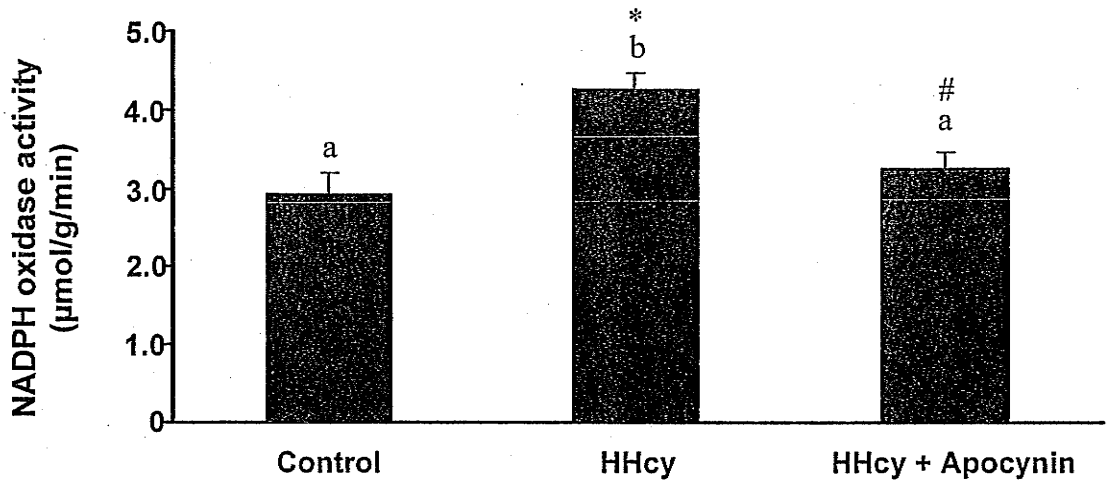


Figure 3. 3. NADPH oxidase activity in the thoracic aortas isolated from the control, HHcy and HHcy + Apocynin groups

The NADPH oxidase activity was measured. Each bar represents the results obtained from an average of 3 experiments. Results were expressed as mean ± SEM. Values without common letter superscripts are significantly different $P<0.05$. *P<0.05 compared with the value of the control group. #P<0.05 compared with the value of the HHcy group.

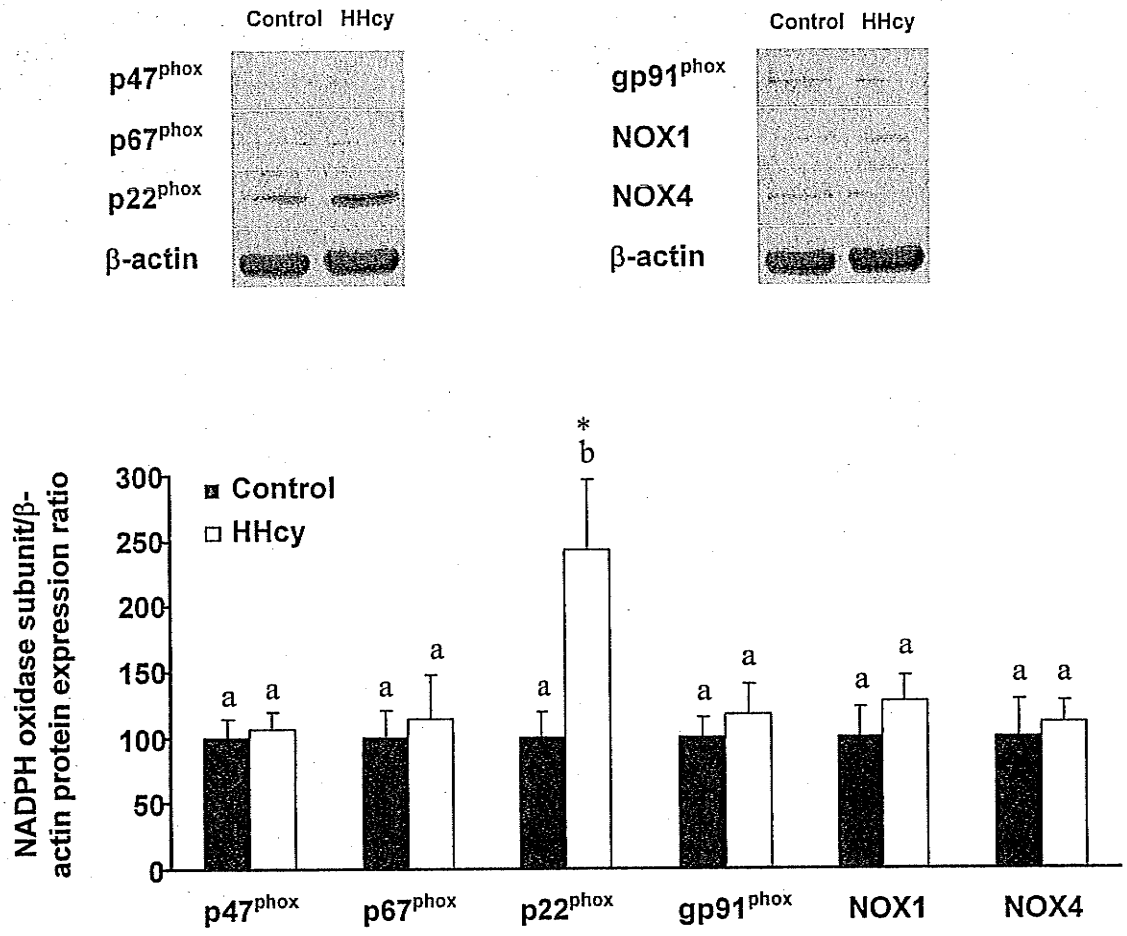


Figure 3. 4. NADPH oxidase protein expressions in thoracic aortas isolated from the control and HHcy groups

The protein levels of $p47^{phox}$, $p67^{phox}$, $p22^{phox}$, $gp91^{phox}$, NOX1 and NOX4 were determined by Western immunoblot analysis. β-actin was used to confirm equal amount of protein loading for each sample. Representative photos were obtained from 3 separate experiments. Results were expressed as mean ± SEM. Values without common letter superscripts are significantly different ($P<0.05$). *$P<0.05$ compared with the control value.

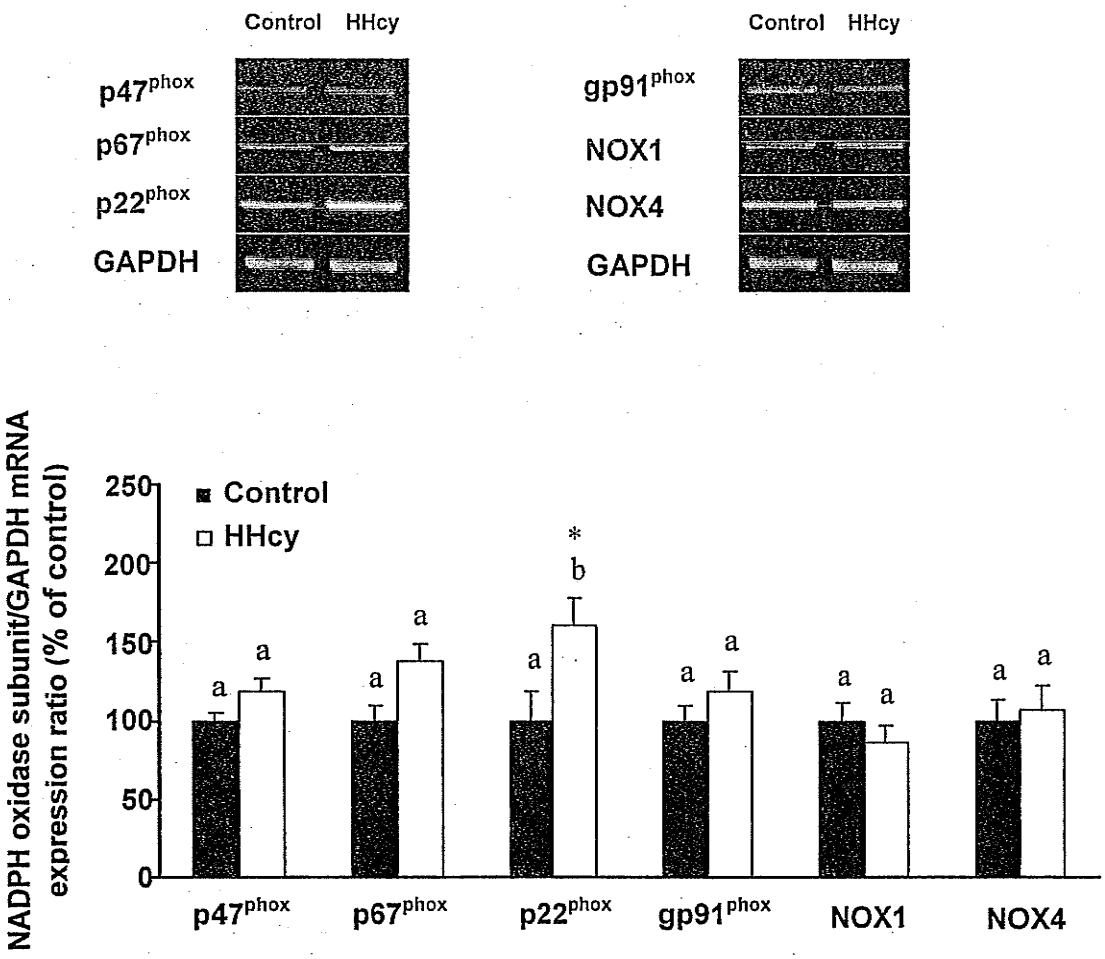


Figure 3. 5. NADPH oxidase mRNA expression in aorta
Total mRNA was isolated. The mRNA levels of individual NADPH oxidase subunits were analyzed by RT-PCR analysis. The GAPDH mRNA was used as an internal standard to verify equal PCR product loading for each sample. Representative photos were obtained from 3 separate experiments. Results were expressed as mean ± SEM. Values without common letter superscripts are significantly different ($P<0.05$). *P<0.05 compared with the value of the control group.

3.1.3 Superoxide Levels, P22^{phox} Expression and p22^{phox} SiRNA Transfection in Vascular Cells

The effect of Hcy on $O_2^{\cdot-}$ production was further examined in cultured endothelial cells (HUVEC). Cells were treated with Hcy (100 μmol/L) for 30 minutes, 2 hours, 4 hours, 6 hours and 12 hours time periods. The intracellular $O_2^{\cdot-}$ production was measured by the NBT reduction assay. The $O_2^{\cdot-}$ levels were significantly elevated in Hcy treated HUVEC (Figure 3.6) after 30 minutes Hcy treatment. There was a significant increase of $O_2^{\cdot-}$ at the 6 hours time point in HUVEC (Figure 3.6) due to gene upregulation of the NADPH oxidase subunit expression.

In the next experiment, HUVEC cells were pre-treated with apocynin for 30 minutes and then incubated with Hcy (100 μmol/L) for another 30 minutes. The intracellular $O_2^{\cdot-}$ production was measured by the NBT reduction assay. The $O_2^{\cdot-}$ levels were significantly elevated in Hcy treated HUVEC at 30 minutes Hcy treatment (Figure 3.7). In the apocynin treated group, the level of $O_2^{\cdot-}$ was reduced to the basal level (Figure 3.7). In the next experiment, HUVEC were pre-treated with apocynin as previously described but incubated with Hcy for another 6 hours. The intracellular $O_2^{\cdot-}$ production was measured by the NBT reduction assay. The $O_2^{\cdot-}$ levels were elevated significantly in Hcy treated cells (Figure 3.8). Next, we investigated whether mRNA level of p22^{phox} subunit of NADPH oxidase was changed in vascular cells by the method RT-PCR. There was a significant increase in p22^{phox} mRNA levels in Hcy treated HUVEC (Figure 3.9) and VSMC (Figure 3.9).

To further examine the link between NADPH oxidase activation and Hcy induced $O_2^{\cdot-}$ production in vascular cells, both HUVEC and VSMC were transfected with p22^{phox}

small interference RNA ($p22^{phox}$ siRNA). Inhibition of NADPH oxidase activation by $p22^{phox}$ siRNA transfection abolished Hcy induced $O_2^{\cdot-}$ production in HUVEC (Figure 3.10) and VSMC (Figure 3.11). These results suggested that NADPH oxidase activation was involved in Hcy induced $O_2^{\cdot-}$ production in vascular cells.

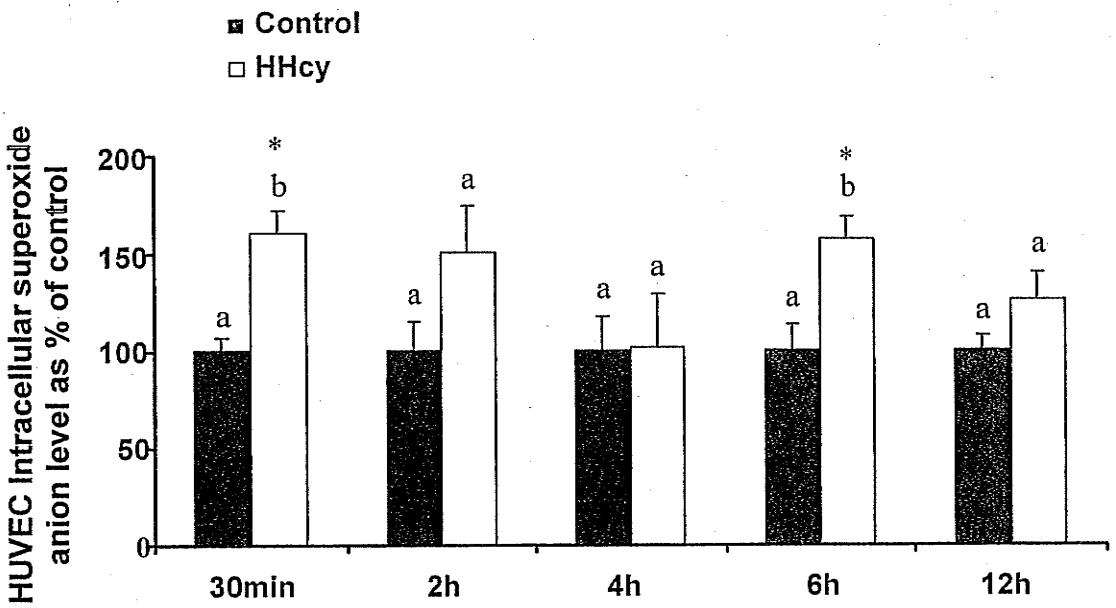


Figure 3. 6. Intracellular superoxide anion levels in HUVEC as a function of the duration of treatment with Hcy

Cells were treated with in the absence (control) and the presence of 100 μmol/L, Hcy and the intracellular $O_2^{\cdot-}$ production was measured by the NBT reduction assay at 30 minutes, 2 hours, 4 hours, 6 hours and 12 hours time points. Results were obtained from 3 separate experiments. Results were expressed as mean ± SEM. Values without common letter superscripts are significantly different ($P<0.05$). *P<0.05 compared with the value of the control group.

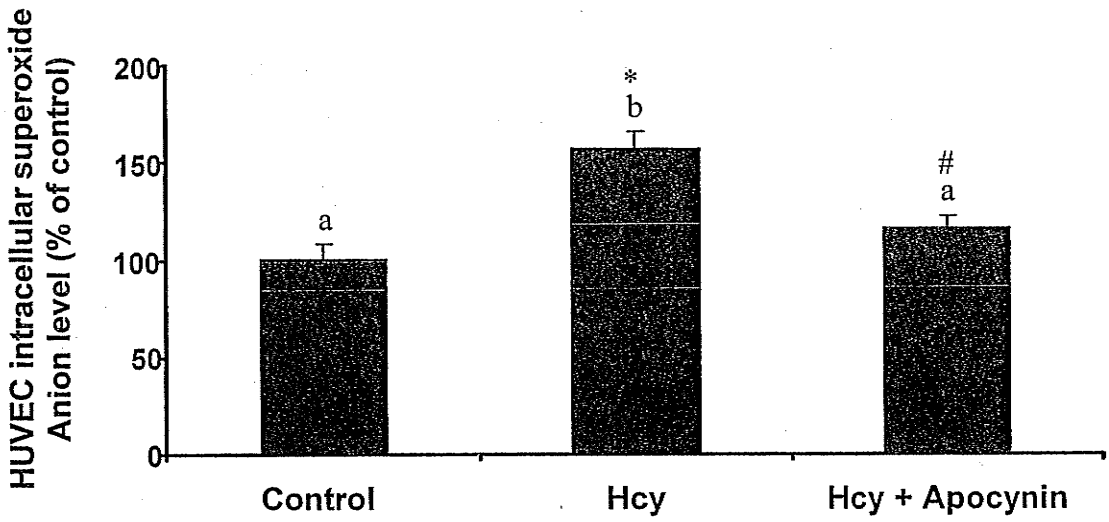


Figure 3. 7. Intracellular superoxide anion levels in HUVEC incubated for 30 minutes

Cells were pre-treated with 300 μmol/L apocynin for 30 minutes and treated with 100 μmol/L, Hcy for 30 minutes and the intracellular $O_2^{\cdot-}$ production was measured by the NBT reduction assay. Results were obtained from 3 separate experiments. Results were expressed as mean ± SEM. Values without common letter superscripts are significantly different ($P<0.05$). *P<0.05 compared with the value of the control group. #P<0.05 compared with the value of the HHcy group.

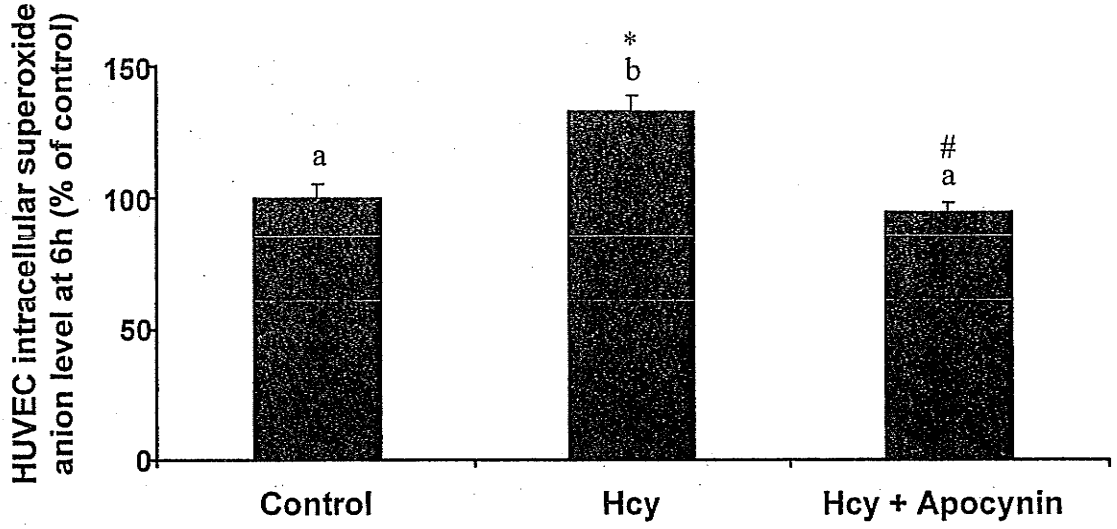


Figure 3. 8. Intracellular superoxide anion levels in HUVEC incubated for 6 hours Cells were pre-treated 300 μmol/L apocynin for 2 hours, treated with 100 μmol/L, Hcy for 4 hours and the intracellular $O_2^{\cdot-}$ production was measured by the NBT reduction assay. Results were obtained from 3 separate experiments. Results were expressed as mean ± SEM. Values without common letter superscripts are significantly different ($P<0.05$). *P<0.05 compared with the value of the control group. #P<0.05 compared with the value of the HHcy group.

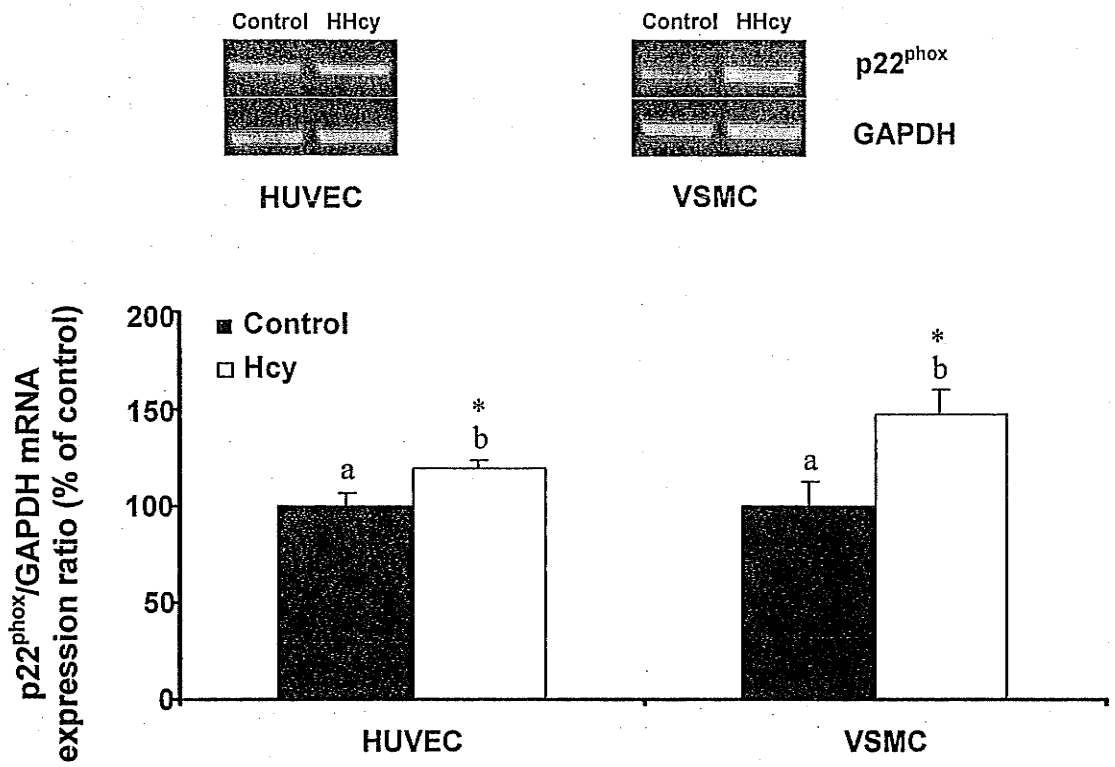


Figure 3. 9. $p22^{phox}$ subunit mRNA expression in HUVEC and VSMC
Total cellular mRNA was isolated and $p22^{phox}$ mRNA in HUVEC and VSMC was measured by a RT-PCR analysis. The GAPDH mRNA was used as an internal standard to verify equal PCR product loading for each experiment. Results were expressed as mean ± SEM. Values without common letter superscripts are significantly different ($P<0.05$). *$P<0.05$ compared with the value of the control group.

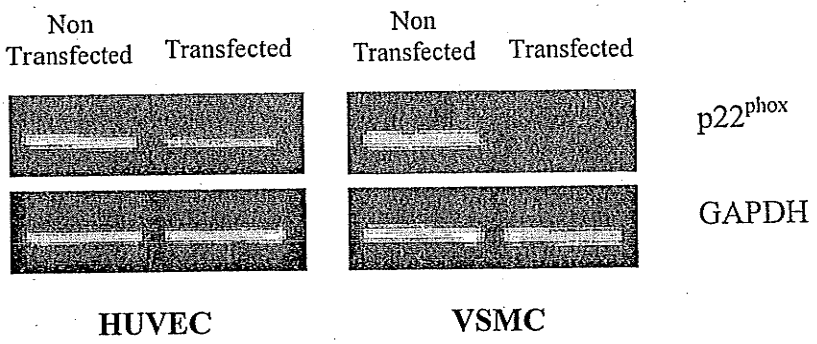


Figure 3.10 mRNA expression of $p22^{phox}$ siRNA transfected HUVEC and VSMC
HUVEC were transfected with $p22^{phox}$ siRNA or a negative control siRNA. At 48 hours. The mRNA expression of the $p22^{phox}$ was measured by RT-PCR.

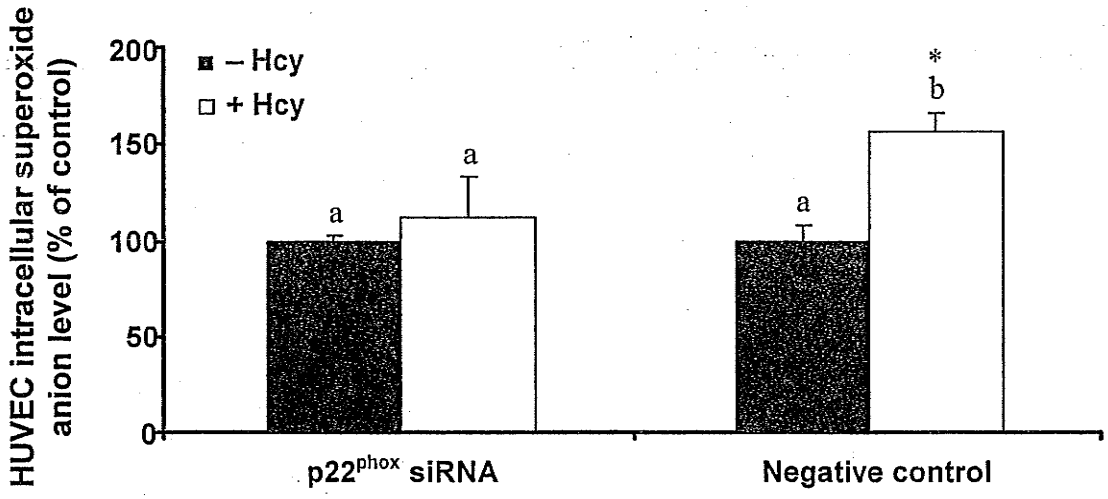


Figure 3. 11. Transfection of endothelial cells with $p22^{phox}$ siRNA
HUVEC were transfected with $p22^{phox}$ siRNA or a negative control siRNA. At 48 hours after transfection, cells were incubated in the presence or absence of Hcy (100 μmol/L) for 6 hours. Intracellular $O_2^{\cdot-}$ were measured by the NBT reduction assay. Results were expressed as mean ± SEM. from three separate experiments each performed in duplicate. Values without common letter superscripts are significantly different ($P<0.05$). *P<0.05 compared with the value of the control group.

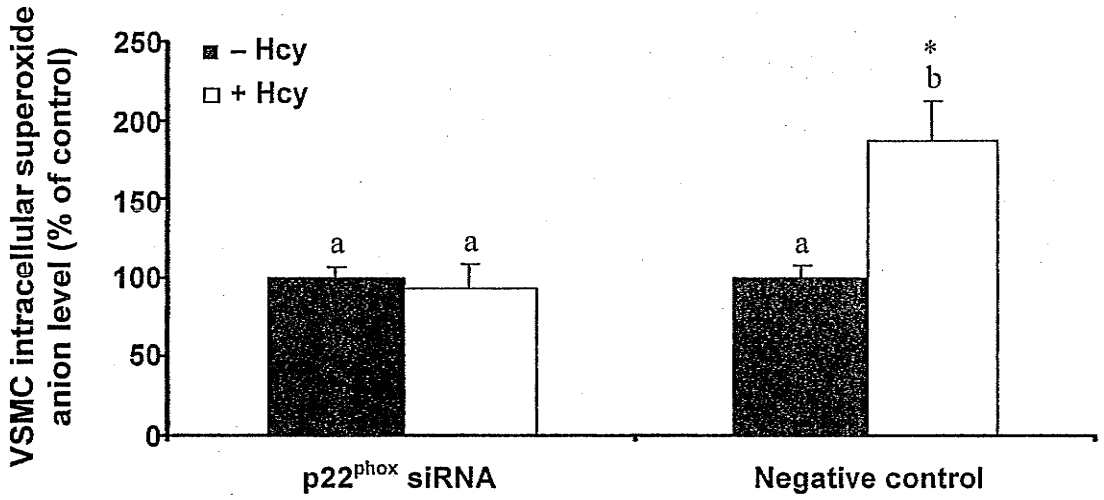


Figure 3. 12. Transfection of vascular smooth muscle cells with $p22^{phox}$ siRNA
VSMC were transfected with $p22^{phox}$ siRNA or a negative control siRNA. At 48 hours after transfection, cells were incubated in the presence or absence of Hcy (100 μmol/L) for 4 hours. Intracellular $O_2^{\cdot-}$ were measured by the NBT reduction assay. Results were expressed as mean ± SEM. from three separate experiments each performed in duplicate. Values without common letter superscripts are significantly different ($P<0.05$). *P<0.05 compared with the value of the control group.

3. 2 Hyperhomocysteinemia and Endothelial Dysfunction

3.2.1 Assessment of the Relaxation of Aorta and Peroxynitrite Levels

The effect of HHcy on endothelial function was examined by measuring the response of aortic rings to acetylcholine or sodium nitroprusside. Endothelium-dependent relaxation to acetylcholine was significantly reduced in the aortas of HHcy rats when compared to that of the control group (Figure 3.12a). Administration of apocynin, an inhibitor for NADPH oxidase, to HHcy rats restored endothelium-dependent relaxation to that of the control level (Figure 3.12b).

The $ONOO^-$ is a potent $O_2^{\cdot-}$ anion-derived oxidant which is involved in different types of tissue injuries (1, 2). To determine whether there was an increase in $ONOO^-$ formation in the aorta during HHcy, immunohistochemical analysis was performed to detect nitrotyrosine, a biomarker for the presence of $ONOO^-$ (Figure 3.13a). There was a marked increase in the intensity of nitrotyrosine protein adduct staining in the aortas of HHcy rats as compared to the control rats (Figure 3.13a). Treatment of HHcy rats with apocynin reduced the level of nitrotyrosine protein adducts to that similar to the control group, indicating that inhibition of NADPH oxidase reversed HHcy induced $ONOO^-$ formation in the aorta (Figure 3.13b). These results suggested that the NADPH oxidase system might be involved in Hcy-induced $O_2^{\cdot-}$ production and $ONOO^-$ formation in the aorta.

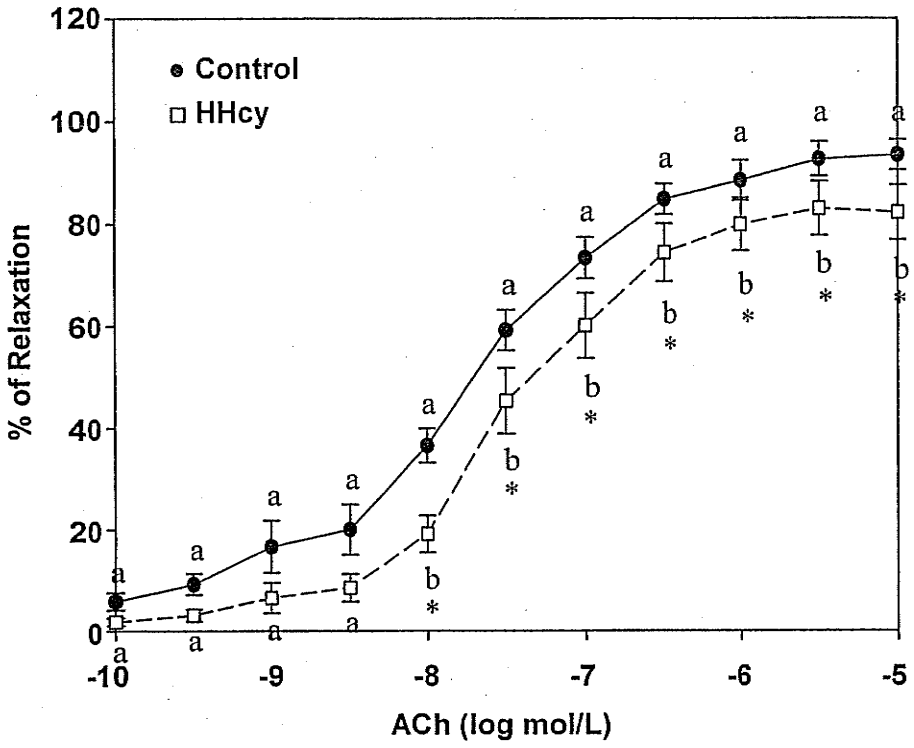


Figure 3. 13a. Endothelium-dependent relaxation of aorta in control and HHcy groups

Thoracic aortas were isolated from the control, HHcy and high methionine groups with apocynin treatment (IP, 4 mg/kg) for 7 days prior to euthanasia. Endothelium-dependent relaxation response to cumulative doses of acetylcholine (ACh) in rat aortic segments was examined using isometric myograph system. Each point represents an average of 5 experiments. Results were expressed as mean ± SEM. Values without common letter superscripts are significantly different ($P<0.05$). *$P<0.05$ compared with the value of the control group.

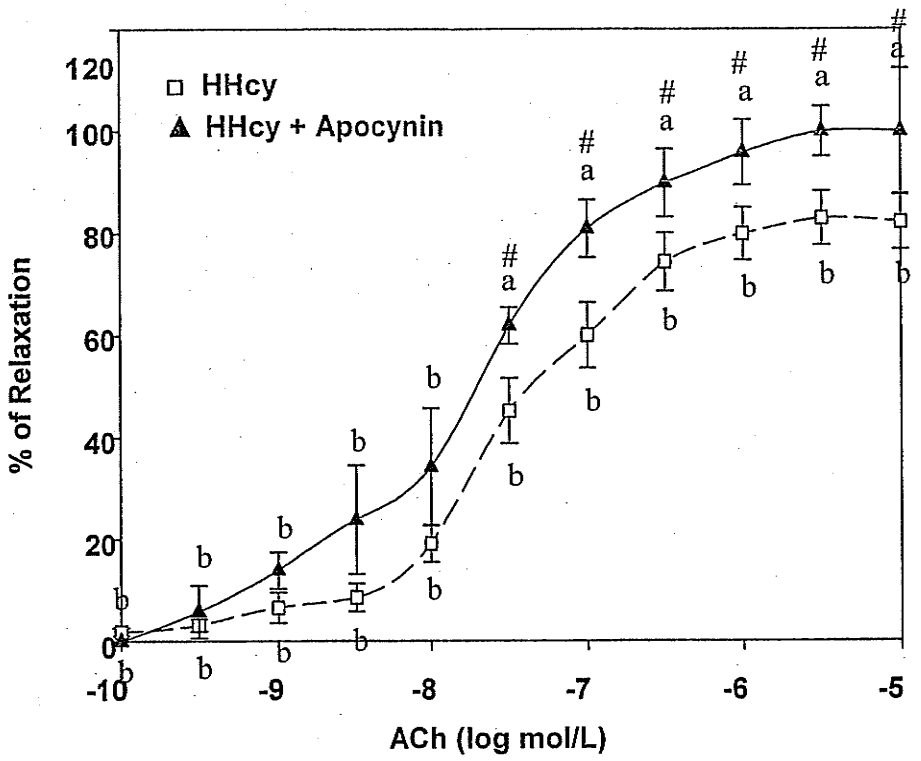


Figure 3. 13b. Endothelium-dependent relaxation of aorta in HHcy and HHcy + apocynin groups

Thoracic aortas were isolated from the control group, high-methionine fed group (HHcy) and high-methionine with apocynin treatment (IP, 4 mg/kg) for 7 days prior to euthan asia. Endothelium-dependent relaxation response to cumulative doses of acetylcholine (ACh) in rat aortic segments was examined using isometric myograph system. Each point represents an average of 5 experiments. Results were expressed as mean ± SEM. Values without common letter superscripts are significantly different ($P<0.05$). #$P<0.05$ compared with the value of the HHcy group.

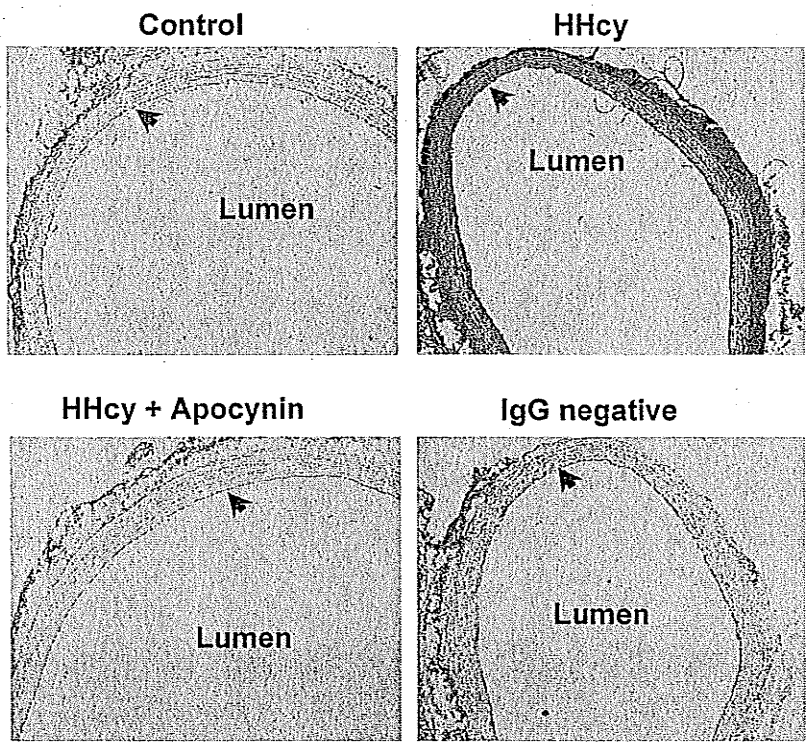


Figure 3. 14a. Nitrotyrosine staining in thoracic aortas isolated from the control, high-methionine fed (HHcy) and (HHcy + Apocynin) groups
Immunohistochemical staining for nitrotyrosine protein adducts was performed with mouse anti-nitrotyrosine antibodies. After counterstaining with Mayer's hematoxylin, nitrotyrosine was identified under light microscope with a magnification of 200 x. Representative photos were obtained from 5 separate experiments. As a negative control, immunohistochemical staining was performed by using non-specific mouse IgG as primary antibodies.

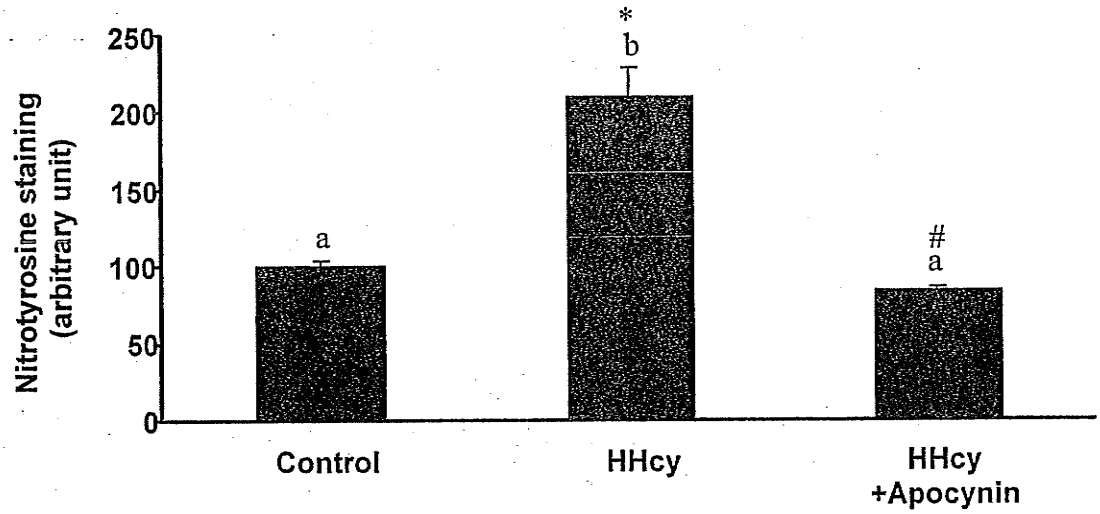


Figure 3. 14b. Determination of the levels of nitrotyrosine in aorta
The intensity of stained area for nitrotyrosine was quantified using Photoshop 6.0. Results were expressed as mean ± SEM. Values without common letter superscripts are significantly different ($P<0.05$). *$P<0.05$ compared with the value of the control group. #$P<0.05$ compared with the value of the HHcy group.

3.2.2 Nitric Oxide Levels and Endothelial Nitric Oxide Synthase Expression in Aorta and Vascular Cells

The impaired endothelium-dependent relaxation might be because of the decrease in endothelial NO· production due to reduced expression of eNOS. Therefore, eNOS mRNA (Figure 3.15) and protein levels (Figure 3.16) were determined in aorta. There were no significant differences in eNOS mRNA (Figure 3.16) and protein (Figure 3.15) levels between the control and HHcy groups of aorta.

The eNOS mRNA (Figure 3.17) and protein (Figure 3.18) expression of HUVEC in the absence (control) or presence of Hcy (100 μmol/L) for 6 hours was measured. There was no significant change either in eNOS mRNA or protein expression. NO· levels of the HUVEC were determined by measuring total nitrates and nitrites in the incubation medium by Griess reaction. HUVEC cells were incubated in the absence (control), or presence of Hcy (100 μmol/L) for 30 minutes and 6 hours. Another set of Hcy treated cells were pre-incubated with 300 μmol/L apocynin. After 30 minutes of Hcy treatment nitrite levels were not changed in the HUVEC compared with the control (Figure 3.19). However, after 6 hours of Hcy treatment, the nitrite levels were significantly reduced in Hcy treated cells, and apocynin pre-treatment was able to bring up the nitrite levels to control level (Figure 3.20).

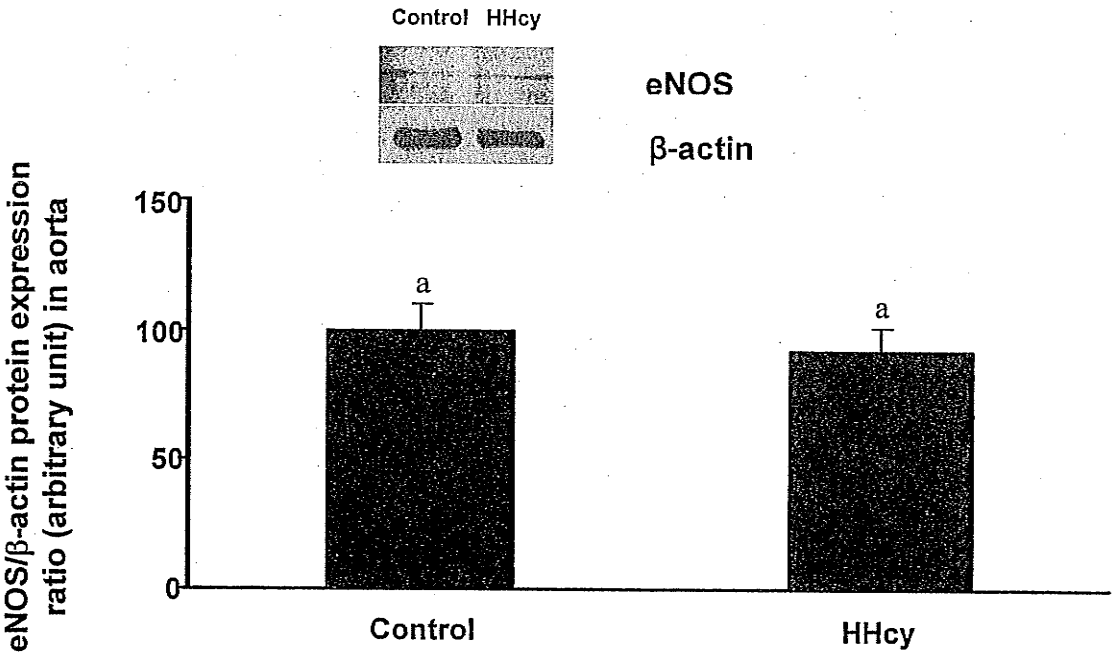


Figure 3. 15. Determination of eNOS protein levels in aorta
Aortas were collected from the control group and the 1.7% methionine fed group (HHcy). The protein levels of eNOS were measured by western immunoblot analysis. Each bar represents an average of 3 experiments. Results were expressed as mean ± SEM. Values with common letter superscripts are not significantly different ($P<0.05$).

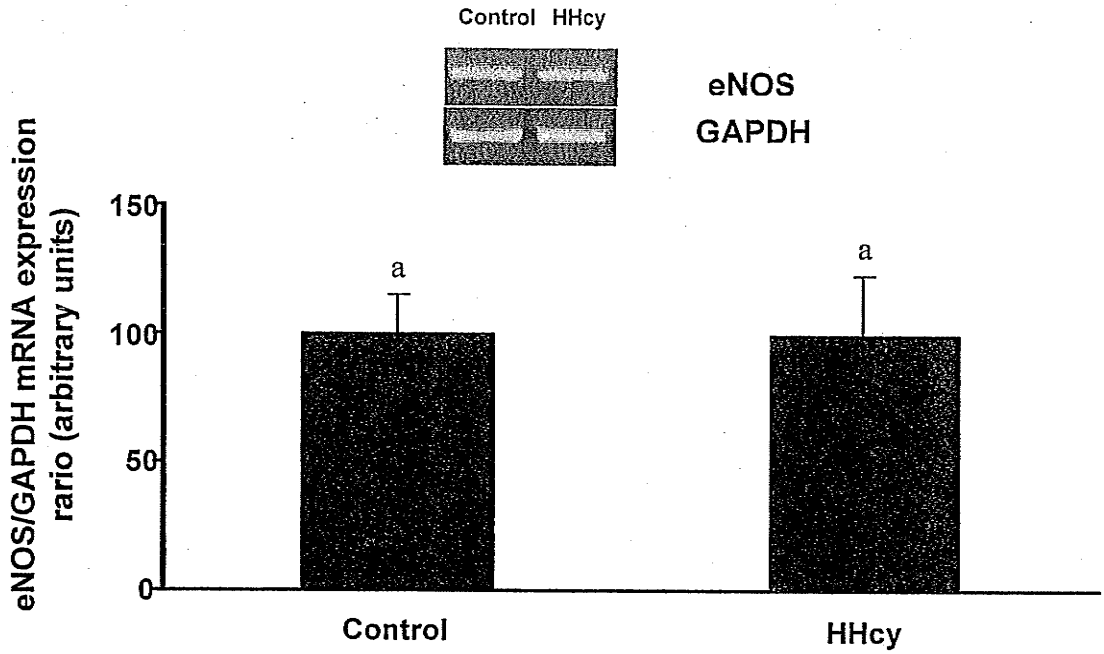


Figure 3. 16. Determination of eNOS mRNA expression in the aorta
Aortas were collected from the control group and the 1.7% methionine fed group (HHcy). The mRNA levels of eNOS were determined by RT-PCR analysis. Each bar represents the results obtained from an average of three separate experiments. Results were expressed as mean ± SEM. Values with common letter superscripts are not significantly different ($P<0.05$).

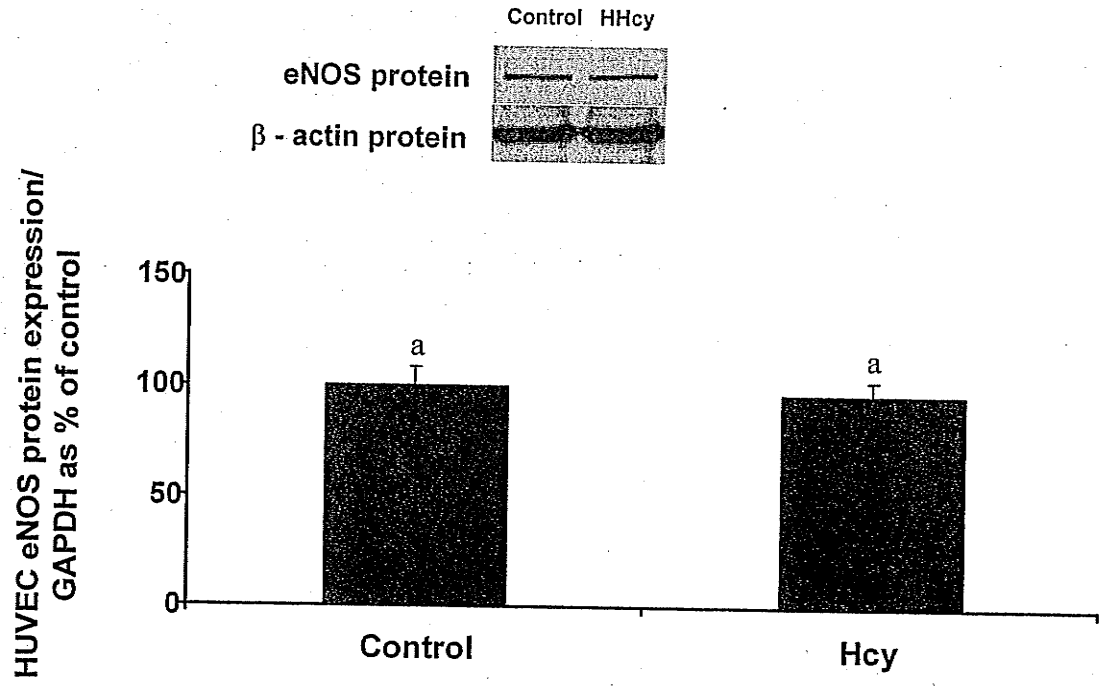


Figure 3. 17. Determination of eNOS protein expression in HUVEC
Cells were treated in the absence (control) or presence of Hcy (100 μmol/L) The protein levels of eNOS were determined by western immunoblot analysis. Each bar represents the results obtained from an average of three separate experiments each performed in duplicate. Results were expressed as mean ± SEM. Values with common letter superscripts are not significantly different ($P<0.05$).

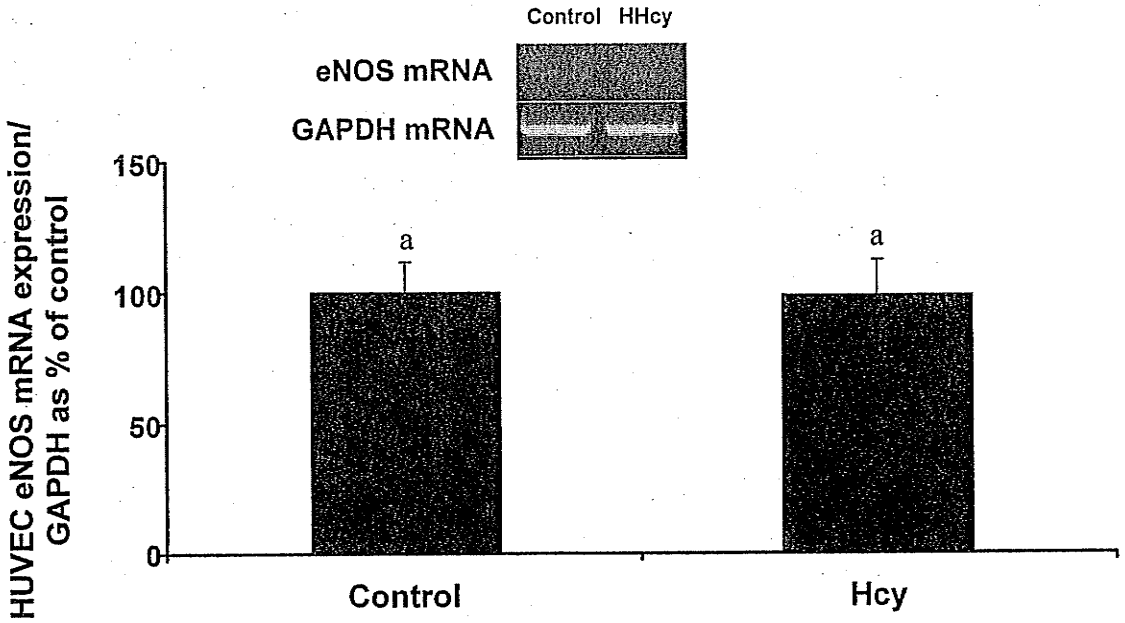


Figure 3. 18. Determination of eNOS mRNA expression in HUVEC
HUVEC cells were treated in the absence (control) or presence of Hcy (100 μmol/L). The mRNA levels of eNOS were determined by RT-PCR analysis. Each bar represents the results obtained from an average of three separate experiments. Results were expressed as mean ± SEM. Values with common letter superscripts are not significantly different ($P<0.05$).

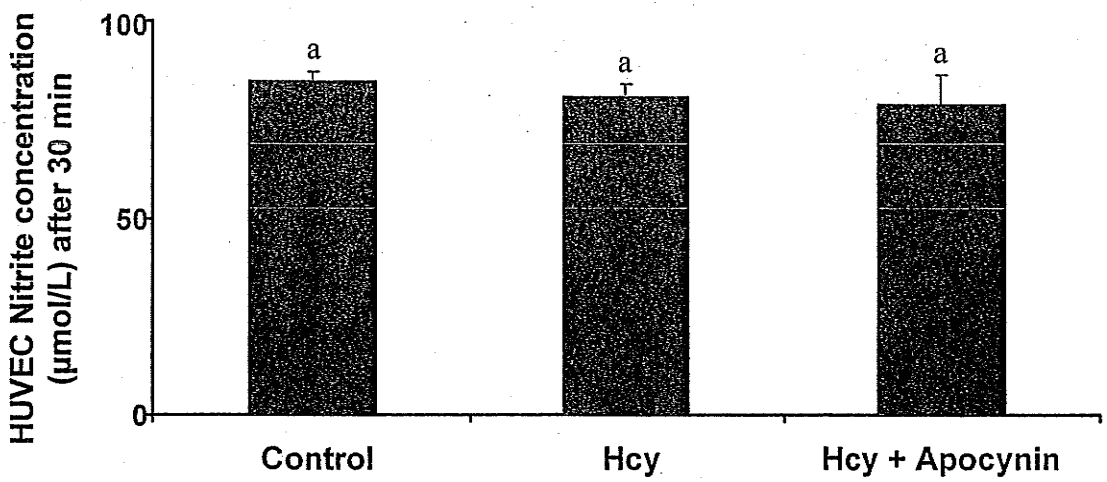


Figure 3. 19. Total nitrite levels in HUVEC after 30 minutes incubation
HUVEC cells were treated in the absence (control) or presence of Hcy (100 µmol/L) for 30 minutes. In one set of experiments, cells were pre-incubated with apocynin (300 µmol/L) before incubating with Hcy. Total nitrite levels in the culture medium were measured by the Griess reaction. Results were expressed as mean ± SEM. Each bar represents the results obtained from an average of three separate experiments each performed in duplicate. Values with common letter superscripts are not significantly different ($P<0.05$).

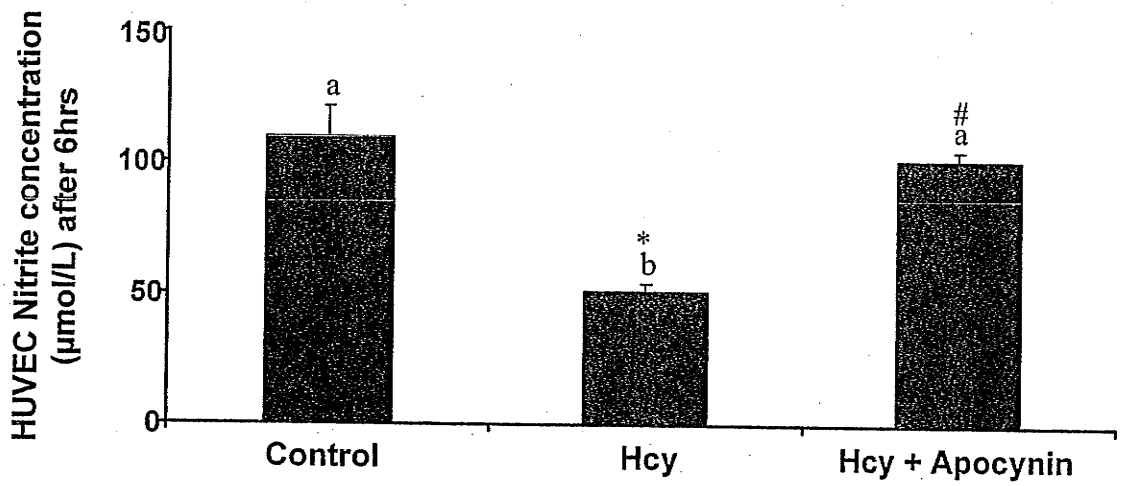


Figure 3. 20. Total nitrite levels in HUVEC after 6 h incubation
HUVEC cells were incubated in the absence (control) or presence of Hcy (100 µmol/L) for 6 h. In one set of experiments cells were pre-incubated with apocynin (300 µmol/L) before incubating with Hcy. Total nitrite levels in the culture medium were measured by the Griess reaction. Results were expressed as mean ± SEM. Each bar represents the results obtained from an average of three separate experiments each performed in duplicate. Values without common letter superscripts are significantly different ($P<0.05$). *$P<0.05$ compared with the value of control group. #$P<0.05$ compared with the value of HHcy group.

CHAPTER 4

DISCUSSION

4.1 Elevation of NADPH Oxidase Mediated Oxidative Stress during HHcy

Hcy, an independent risk factor for CVD, exerts its adverse effects on vasculature by several mechanisms. One of the mechanisms is oxidative stress (Au-Yeung et al. 2004). A previous publication shows that elevated Hcy levels have a significant stimulatory effect on the ROS production in vessels (Au-Yeung et al. 2004). However, the mechanism by which Hcy induced oxidative stress was not clearly established. In this study, we have identified an important mechanism of Hcy induced oxidative stress in the upregulation of the NADPH oxidase system. By employing several lines of *in vivo* and *in vitro* methods, we have demonstrated that Hcy induced $O_2^{\cdot-}$ production is invariably linked with NADPH oxidase activity.

The first part of the study was focused on the ROS production during HHcy. Initially, the total Hcy level in rat serum was measured to test the validity of the HHcy animal model. The serum Hcy levels in the HHcy rats showed a significant elevation over the serum Hcy levels in the control rats. One set of HHcy rats that were injected with NADPH oxidase inhibitor, apocynin did not show a variation of serum Hcy levels from the HHcy group. It indicated that injection of apocynin had no effect on the serum Hcy levels, suggesting that the effects showed by the apocynin injected HHcy group was solely due to the specific action of apocynin on NADPH oxidase.

The total $O_2^{\cdot-}$ production in the aorta was measured to detect whether HHcy had an upregulatory effect on the $O_2^{\cdot-}$ production in the animal model. The HHcy rat aortas showed significant elevation in $O_2^{\cdot-}$ level compared with the control aortas. This observation concurred with previous data on HHcy and ROS production reported by our laboratory (Au-Yeung et al. 2004). The apocynin injected HHcy group showed a

significant reduction in $O_2^{\cdot-}$ levels compared with the HHcy group. This indicated that $O_2^{\cdot-}$ production during HHcy might be mediated by NADPH oxidase activation.

The NADPH oxidase activity was measured in the aortas isolated from control and HHcy rats. NADPH oxidase activity was significantly higher in the HHcy aortas than that of control aortas. This indicated that elevated serum Hcy levels had an upregulatory effect on the NADPH oxidase system. This upregulation might contribute to the elevated $O_2^{\cdot-}$ levels seen in HHcy rat aorta. If such was the case, Hcy-induced $O_2^{\cdot-}$ production should be reversed by NADPH oxidase inhibitor. As expected the NADPH oxidase activity in the apocynin injected HHcy group was significantly reduced versus the HHcy group. This indicated that the elevated NADPH oxidase activity during HHcy was indeed responsible for the increased oxidative stress in the aorta.

To investigate the effect of HHcy on the expression of NADPH oxidase subunits, both the protein and mRNA levels were measured. The subunit $p22^{phox}$ protein level was significantly increased in HHcy aortas compared with control aortas. This observation was compatible with the mRNA expression level of the $p22^{phox}$ subunit. However, the other NADPH oxidase subunits did not show a significant elevation during a HHcy situation. A recent study done with Wistar-Kyoto rats where methionine was added to the drinking water for 4weeks demonstrated the activation of NADPH oxidase in the coronary arteries of HHcy rats. This activation was due to the increased expression of NOX1 (Ungvari et al. 2003). This study did not show an elevation of $p22^{phox}$ expression (Ungvari et al. 2003). The differences seen in the regulation of NADPH oxidase subunit expression in the above study compared with the current study might be due to the difference in the types of blood vessels tested. The resistance arteries such as the

coronary artery have considerably different regulatory mechanisms in terms of vascular function than conduit arteries such as the aorta. These differences in the regulation of vascular function might also apply to the regulation of NADPH oxidase subunit activation during HHcy (Szasz et al. 2007).

Another study demonstrated that during HHcy, cardiac $p22^{phox}$ expression was up regulated in Wistar-Kyoto rats given methionine in drinking water (Becker et al. 2005). The Hcy upregulated cardiac $p22^{phox}$ expression led to increased NADPH oxidase subunit assembly and activation (Becker et al. 2005). Also, they observed a significant reduction in the expression of $gp91^{phox}$ (Becker et al. 2005). The difference seen in the regulation of $gp91^{phox}$ subunit expression in this study compared to the current study might be due to the differences between the vascular cells and cardiac cells.

We also investigated whether the *in vivo* observations were reproducible under an *in vitro* situation. HUVEC treated with Hcy both at 30 minutes and 6 hours diplayed significantly higher intracellular $O_2^{\cdot-}$ levels, whereas VSMC treated with Hcy at 30 minutes and 4 hours had the highest values. The elevated $O_2^{\cdot-}$ anion level at 30 minutes might be due to the elevated NADPH oxidase activity due to the subunit assembly. The elevated $O_2^{\cdot-}$ anion levels at 6 hours and 4 hours time points were probably due to the subunit upregulation of the enzyme at the gene expression level. Pre-treatment with apocynin for 30 minutes prior to the incubation with Hcy was able to reverse the $O_2^{\cdot-}$ anion production significantly at 30 minutes in HUVEC but for VSMC 2 hours pre-treatment with apocynin was needed before incubating with Hcy to detect a reversal of $O_2^{\cdot-}$ production. The differences in response to apocynin pre-treatment may be due to the differences between the NADPH oxidase systems found in these two types of vascular cells. Several

studies have shown that vascular cells and other non phagocytic cells respond to *in vitro* apocynin treatment within a diverse range (Riganti et al. 2006; Vejrazka et al. 2005). The effects of apocynin as an NADPH oxidase inhibitor was first tested in phagocytic cells (Stolk et al. 1994; Van den Worm et al. 2001). The $gp91^{phox}$ centered NADPH oxidase system in phagocytes was highly responsive to apocynin mediated inhibition. This inhibitory effect was due to the inhibition of the translocation of cytosolic subunits (Stolk et al. 1994). However, the mechanism of apocynin inhibition of the NADPH oxidase systems in non-phagocytic cells is unclear (Vejrazka et al. 2005). The current study suggests that apocynin has an inhibitory effect on subunit translocation of the NADPH oxidase in HUVEC during *in vitro* situations. $p22^{phox}$ subunit mRNA expression was measured in both types of cells to detect whether Hcy had an upregulatory effect on $p22^{phox}$ subunit. Again, in these cells the $p22^{phox}$ subunit was elevated at mRNA expression level. To further confirm the involvement of NADPH oxidase in Hcy-induced $O_2^{\cdot-}$ production, NADPH oxidase was inhibited by knocking down (silencing) mRNA expression of one subunit of the enzyme. All subunits of NADPH oxidase are needed to form a complete and active enzyme. If even one subunit was not present, the enzyme activation could not take place. Thus, NADPH oxidase mediated $O_2^{\cdot-}$ production could not occur. Transfection of both vascular cells with $p22^{phox}$ short interference ribonucleic acid ($p22^{phox}$ siRNA) inhibited the Hcy-induced $O_2^{\cdot-}$ production in HUVEC and VSMC. This confirmed the hypothesis that Hcy induced $O_2^{\cdot-}$ production (i.e. oxidative stress) was mediated via NADPH oxidase activation.

4.2 Endothelial Dysfunction during HHcy

The second part of the study was focused on the effect of HHcy on endothelial dysfunction. It has been shown by other investigators that HHcy leads to endothelial dysfunction (Abahji et al. 2007; Clarke et al. 2006; Weiss et al. 2002a; Weiss et al. 2002b). In the HHcy rat model used in the current study, endothelial dysfunction was demonstrated by the impaired aortic relaxation compared with the control. Apocynin treatment showed a significant improvement of the relaxation of the aorta. Apocynin was used to reverse the ROS production in aorta in several previous studies (Hcy-mediated and non Hcy-mediated) all of which showed an improvement of endothelium function (Cai and Harrison 2000; Hamilton et al. 2001; Hayashi et al. 2005; Matsumoto et al. 2006; Oelze et al. 2006; Sanchez et al. 2007). These results showed that during HHcy, the endothelial dysfunction was mediated by $O_2^{\cdot -}$ produced by NADPH oxidase. $NO^{\cdot}$ is the regulator of endothelial function in large vessels. To further investigate the mechanism, $ONOO^-$ formation was measured in the aorta. $ONOO^-$ is formed by the combination of $NO^{\cdot}$ with a $O_2^{\cdot -}$ anion. This reaction reduces the bioavailability of $NO^{\cdot}$ by further forming the stable adduct nitrotyrosine with protein tyrosine. $ONOO^-$ formation depicted by nitrotyrosine staining was markedly elevated in the HHcy group of aortas compared with the control aorta. The apocynin treated HHcy group showed a significant reduction of the nitrotyrosine staining compared with the HHcy aorta. This observation suggested that during HHcy the impaired endothelial function was caused by the reduced bioavailability of $NO^{\cdot}$.

4.3 Regulation of eNOS Expression during HHcy

The third part of the study was designed to determine whether HHcy had a downregulatory effect on eNOS expression both at protein expression and mRNA

expression levels. There were no changes in the eNOS mRNA expression or the protein expression during HHcy condition. During HHcy situations, the NO· production was not reduced by the downregulation of the eNOS enzyme. Similar observations were seen during *in vitro* experiments in which endothelial cells were treated with Hcy for 6 hours. The total nitrite levels of HUVEC cells not-treated (control) and treated with Hcy were measured at both 30 minutes and 6 hours. One set of Hcy treated cells were pre-incubated with apocynin for 30 minutes. At 30 minutes there was no difference in the total nitrite levels of Hcy treated or apocynin pre-incubated, Hcy treated HUVEC. However, at 6 hours there was a significant reduction in the nitrite levels in the Hcy treated cells compared with the control cells. Pre-treatment of the cells with apocynin prior to Hcy treatment was able to elevate the nitrite levels up to control levels. These observations taken together with the *in vitro* $O_2^{\cdot-}$ production by the endothelial cells further indicated that prolonged (6 hours) exposure to Hcy leads to reduction of the bioavailability of NO· by the elevated $O_2^{\cdot-}$ production.

4.4 Summary

HHcy leads to elevated ROS ($O_2^{\cdot-}$) production in aorta which creates a pro-oxidative environment in the tissue. This pro-oxidative environment was responsible for the impairment of the endothelium-dependent vasorelaxation observed during HHcy. NADPH oxidase is the main $O_2^{\cdot-}$ producer in vascular tissues. The elevated $O_2^{\cdot-}$ production during HHcy was due to the elevated activity of NADPH oxidase. This conclusion was based upon several experimental observations. First, elevated $O_2^{\cdot-}$ was observed during HHcy which was reversed to the control level by the treatment of apocynin, NADPH oxidase inhibitor. Second, NADPH oxidase activity was also

increased during HHcy which in turn could be reversed by apocynin treatment. Third, the $p22^{phox}$ subunit of the enzyme was elevated at both the protein and mRNA levels in HHcy aorta. The *in vitro* experiments with HUVEC showed that the $p22^{phox}$ subunit expression was similar to that of the aorta. Silencing post transcriptional expression of $p22^{phox}$ subunit inactivated NADPH oxidase. When the transfected cells were treated with Hcy there was no elevation of $O_2^{\cdot-}$ production. This confirms the NADPH oxidase involvement in oxidative stress during HHcy. Elevated $O_2^{\cdot-}$ during HHcy leads to the impaired endothelium-dependent relaxation of aorta. Elevated $ONOO^-$ during HHcy indicated that bioavailability of $NO^{\cdot}$ was reduced. Taken together, these results suggest that endothelial dysfunction during HHcy was due to the upregulation of NADPH oxidase which elevated the $O_2^{\cdot-}$ production that was responsible for the reduced bioavailability of $NO^{\cdot}$.

CHAPTER 5

CONCLUSIONS

5. 1 Conclusions

There were several conclusions drawn from this research.

1) HHcy induced oxidative stress in rat aorta was caused by the elevated $O_2^{\cdot-}$ anion production due to increased NADPH oxidase activity.

2) Hcy-induced $O_2^{\cdot-}$ anion production in vascular cells was mediated via activation of NADPH oxidase due to the upregulation of the $p22^{phox}$ subunit.

3) Endothelial dysfunction in the aorta during HHcy was due to the reduced $NO^{\cdot}$ bioavailability mediated by elevated $O_2^{\cdot-}$ anion production and subsequent $ONOO^{-}$ formation.

4) Endothelial dysfunction observed during HHcy was not due to the downregulation of eNOS expression.

In summary, these results suggest that Hcy-induced $O_2^{\cdot-}$ production via NADPH oxidase and subsequently $ONOO^{-}$ formation contribute to impaired endothelium-dependent vessel relaxation during HHcy. A better understanding of the mechanisms by which Hcy induces endothelial dysfunction would help to develop better strategies for the prevention and treatment of cardiovascular diseases.

5. 2 Future directions

Apart from oxidative stress, Hcy has also been shown to activate transcription factors, signaling cascades and inflammatory factors as well as induce ER stress, which in turn, can lead to endothelial dysfunction (Au-Yeung et al. 2004; Wang et al. 2002; Werstuck et al. 2001). The contributions of those mechanisms to Hcy-induced endothelial dysfunction remain to be investigated in future studies.

Folic acid studies on HHcy subjects revealed that folic acid could reduce the plasma HHcy levels significantly by increasing the remethylation pathway. Reducing Hcy by folic acid treatment could be utilized as an intervention measure.

Once the mechanisms of action of HHcy in relation with CVD are understood, new preventative and intervention therapy for HHcy mediated CVD can be effectively implemented.

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