Assessment of Lipopolysaccharide, Isoproterenol and Phenylephrine Induced Pathophysiology in Lipoic Acid Synthase Deficient Mice

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ABSTRACT

KUIKWON KIM. Assessment of Lipopolysaccharide, Isoproterenol and Phenylephrine Induced Pathophysiology in Lipoic Acid Synthase Deficient Mice (Under the direction of Dr. Nobuyo Maeda.)

α-Lipoic acid is a potent antioxidant, and also an essential cofactor for PDH and α-KDH complexes in mitochondria. Investigations have shown complex human diseases are associated with oxidative stress. As preventative and therapeutic purposes, studies have evaluated effects of lipoic acid on complex human diseases, however, its molecular mechanisms and effects remained unknown.

In this study, we examined the effects of heterozygous Lias deficiency on LPS induced inflammation and adrenergic agonist induced cardiac hypertrophy. We observed increased sensitivity of Lias heterozygous mice with elevation of TNF-α, decreased and delayed recovery of leukocyte and platelet counts accompanied with liver necrosis lung inflammation on LPS stimuli. Upon adrenergic agonist stimulation, both genotypes developed hypertrophy with increased HW/BW ratio and fibrosis in the heart, however, we observed protective effect of Lias deficiency upon stimulation. Our data indicates that Lias deficiency results in increased sensitivity upon LPS stimulation, however, protective effect against cardiac stimulation.

ACKNOWLEDGEMENTS

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ABBREVIATIONS

ALT	Alanine Aminotransferase
ANP (ANF)	Atrial Natriuretic Peptide (Factor)
AST	Aspartate Aminotransferase
BSA	Bovine Serum Albumin
BUN	Blood Urea Nitrogen
cAMP	Cyclic Adenosine Monophosphate
DHLA	Dihydrolipoic Acid
GRO	Growth Related Oncogene
GSH	Reduced Glutathione
I-CAM	Inter Cellular Adhesion Molecule
IL-10	Interleukin-10
IL-6	Interleukin-6
iNOS	Inducible Nitric Oxide Synthase
IP	Intraperitoneal
ISO	Isoproterenol
LA	Lipoic Acid
LIAS	Lipoic Acid Synthase
LPS	Lipopolysaccharide
MCAD	Medium Chain Acyl-CoA Dehydrogenase
MCP-1	Monocyte Chemoattractant Protein-1
MDA	Malondialdehyde

MIP-1α	Macrophage Inflammatory Protein-1 Alpha
Mn-SOD	Manganese Superoxide Dismutase
MPO	Myeloperoxidase
NF-κβ	Nuclear Factor Kappa B
NO	Nitric Oxide
PBS	Phosphate Buffered Saline
PDH	Pyruvate DeHydrogenase
PE	Phenylephrine
PFA	Paraformaldehyde
ROS	Reactive Oxygen Species
TBARS	Thiobarbituric Acid Reactive Substances
TNF-α	Tumor Necrosis Factor-alpha
V-CAM	Vascular Cell Adhesion Molecule
WBC	White Blood Cell
α-KDH	Alpha-Ketogulterate DeHydrogenase
β-MHC	Beta Myosin Heavy Chain

CHAPTER 1

INTRODUCTION

1.1 OXIDATIVE STRESS AND COMPLEX HUMAN DISEASES

Diabetes, cardiovascular disease, and hypertension are important and complex human diseases which are major causes of mortality and morbidity in the world today. Investigations have revealed several causative factors of these complex human diseases: genetic factors, ethnic background, gender, nutrition, sedentary life styles, and aging (Smith, 2007). A concept of oxidative stress and reactive oxygen species has been under extensive debate as a contributing factor to the initiation and progression of diabetes, cardiovascular diseases and hypertension. Oxidative stress, caused by ROS (reactive oxygen species), is defined as any cellular damage resulting from an imbalance between an excessive generation of oxidant compounds and insufficient antioxidant defense mechanisms (Sies *et al*., 1997).

Oxidative stress may damage cellular macromolecules. For example, oxidative stress can biochemically modify proteins and lipids, and often leads to modification and fragmentation of amino acid sequences and lipid structures. These modifications result in alterations and loss of properties as functional products in the biological system (Cecarini *et al*., 2007; Bartsch *et al*., 2006). Moreover, oxidative damage, much like ionizing radiation on DNA, is known to cause deletion, insertion, inversion and translocation in base pairs, and

consequently leads to DNA degradation, single strand breakage and cellular damage (Imlay *et al*., 1998).

Currently, many clinical and animal models of investigations address issues of the reactive oxygen species as one of causative factor, in diabetic complications such as atherosclerosis, nephropathy, and polyneuropathy. These studies suggest that antioxidant supplements may have preventive and therapeutic effects on these diseases. The outcome of these clinical tests are, however, consistently positive. Nevertheless, genetic alterations in the genes controlling the body's antioxidant defense system is another potential candidate that may influence the risk for developing these diseases (Wollin *et al*., 2003; Maritim *et al*., 2003; Opara *et al*., 2002; Gotto, 2003; Ziegler *et al*., 2004).

1.2 ANTIOXIDANT AND LIPOIC ACID

According to Halliwell and Gutteridge (1989), an antioxidant is defined as any substance that delays or inhibits oxidation of oxidizable substrates, including any molecular compounds. Every living organism produces endogenous oxidants as a form of aerobic metabolism and endogenous buffering systems naturally balance oxidants and free radicals by initiating antioxidant defense mechanisms. Sies *et al*. summarizes and describes three principle functions of antioxidant defense mechanisms - prevention, interception, and repair (Sies *et al*., 1997).

Prevention of endogenous oxidative stress from oxidative metabolism can be achieved by several enzymatic activities. Removing or transferring radicals and enzymes including glutathione S-transferases, plays a role of prevention. Strategies of interception of free radicals and oxidative stress also play an important role in transferring damaging

radicals away from sensitive cells and tissues. Several enzymatic reactions and non-enzymatic molecular compounds are responsible for intercepting harmful free radicals including-vitamin E, carotenoids, superoxide dismutase, catalases and glutathoione peroxidases. Elevated rates of harmful radicals and accumulation of oxidants under certain physiological conditions may damage structures of DNA, proteins and lipids. However, these molecular and cellular damages can be repaired by specific repair mechanisms or substituted by multiple lipolytic and proteolytic enzyme complexes (Sies *et al*., 1997).

In the last decade, alpha-lipoic acid (LA or 6, 8-thioctic acid) gained much attention as a potent and powerful antioxidant compound because of its preventive and therapeutic effects on many types of complex human diseases, including diabetes, atherosclerosis, and neurodegenerative diseases (Packer *et al*., 2001; Bilska and Wlodek, 2005; Bielarczyk *et al*., 2006; Ziegler *et al*., 2004), (Figure 1.1.).

Lipoic acid (LA) is a naturally occurring compound and a crucial disulfide/dithiol cofactor for pyruvate dehydrogenase (PDH), and alpha-ketogluterate dehydrogenase (α-KDH), which are important multienzyme complexes in energy metabolism in mitochondria (Reed *et al*., 1951), (Figure 1.2.). As one of the most potent antioxidants, LA and its reduced form DHLA, have drawn much interest because of its several unique features (Packer *et al*., 2001; Moini *et al*., 2002). First, LA/DHLA is an amphiphilic compound and easily crosses the blood brain barrier. Second, it can quench free radicals and chelate heavy metals. Third, it interacts with other antioxidants such as vitamin C, vitamin E, glutathione coenzyme Q10, and ubiquinone, and metabolically can be regenerated by other antioxidants (Gotz *et al*., 1994; Han *et al*., 1997; Kozlov *et al*., 1999; Packer *et al*., 1995; Scholich *et al*., 1989).

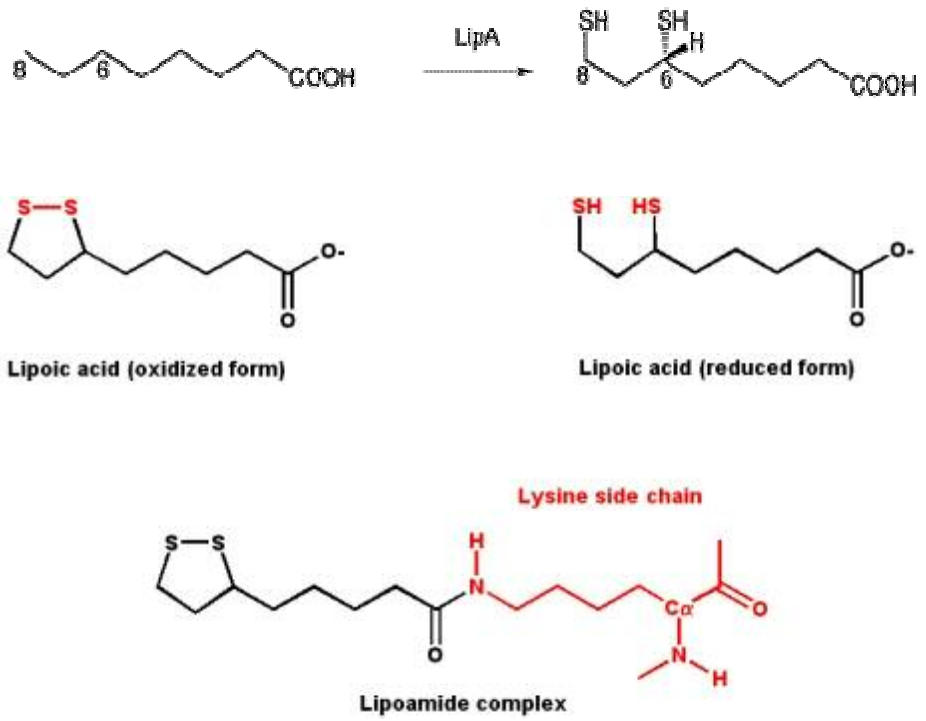

Figure 1.1. Molecular structures of lipoic acid. Lipoic acid is synthesized from octanoic acid and an unknown sulfur source in cell by lipoic acid synthase. Lipoic acid exists in two forms, oxidizied and reduced which both act as strong antioxidants. Naturally, lipoic acid is formed as lipoamide complex (lysine side chain). (Bilska and Wlodek, 2001; Packer *et al*., 2001, 2002; Moini *et al*., 2002).

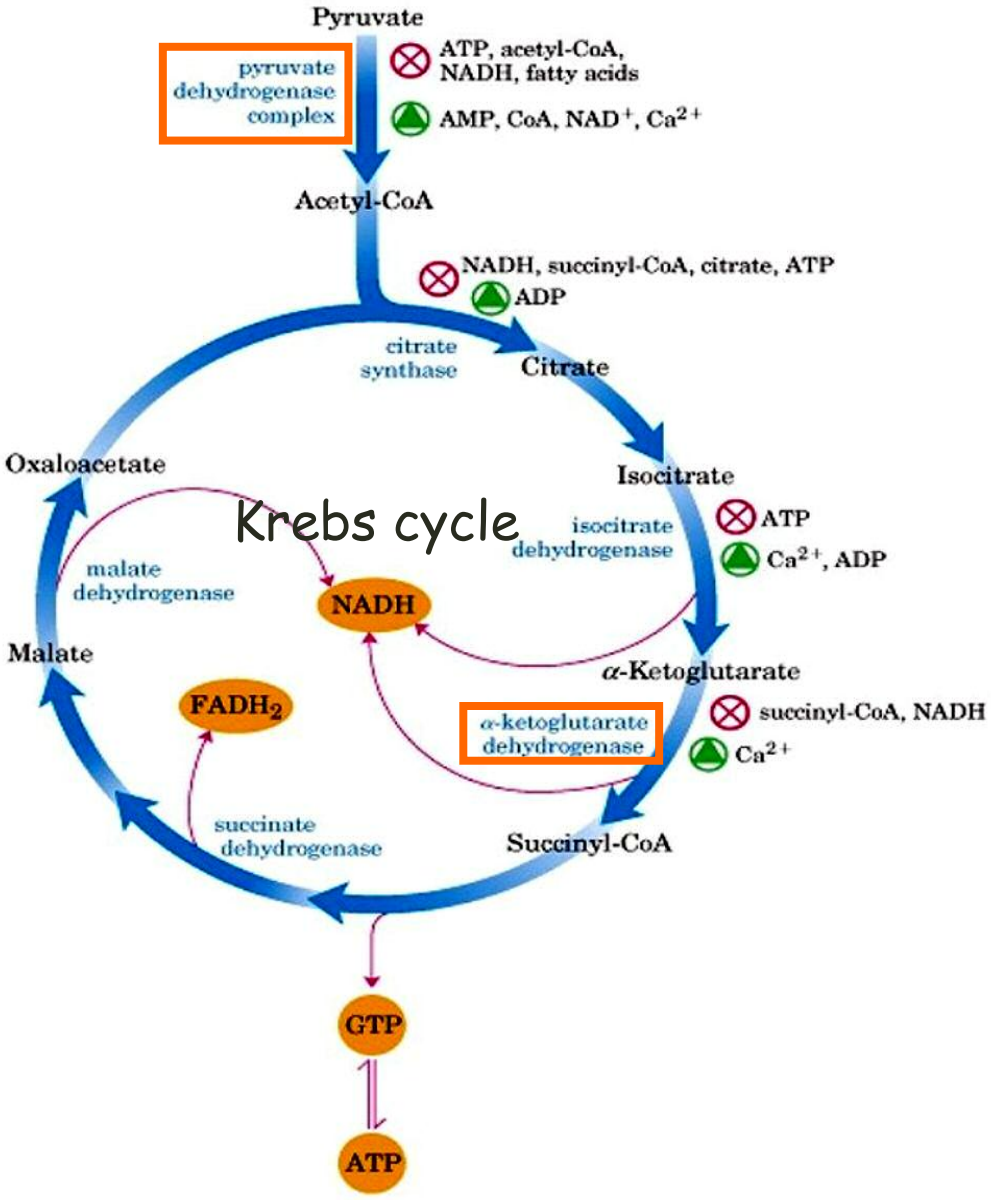

Figure 1.2. Oxidative metabolism in mitochondria and lipoic acid. Pyruvate is transported into mitochondria and acetyl-CoA is synthesized by pyruvate dehydrogenase complex. TCA cycle begins with transferring two carbons, acetyl CoA, to four carbon acceptor, oxaloacetate to form six carbon citrate. This Citrate goes through a series of cycles of reducing its carbon number and producing NADH, $FADH_2$, CO_2 and GTP. Lipoic acid is a crucial cofactor for pyruvate dehydrogenase and alpha-ketogluterate dehydrogenase (Created based on the Marcotte, Fall, 2005 Chapter 16, Citric Acid Cycle)

1.3 LIPOIC ACID SYNTHASE AND MOUSE MODEL OF DEFICIENCY

Lipoic acid is universally present in prokaryotes and eukaryotes, and its endogenous production is regulated by lipoic acid synthase (*Lias*) from octanoic acid and an unknown sulfur source (Packer *et al*., 2001; Jordan *et al*., 1997; Miller *et al*., 2000). The lipoic acid synthase gene is shared by many species (Morikawa *et al*., 2001), and is encoded by the nuclear gene *Lias* located on mouse chromosome *5*.

Since it has been shown that LA plays crucial roles as a potent antioxidant in several biological systems, and as a critical cofactor of multi enzyme complexes that are important in energy metabolism, Lias is a potential candidate gene for cardiovascular and metabolic diseases. To test this hypothesis, our laboratory generated mice deficient in lipoic acid synthase. We did this in order to explore the genetic and physiological effects of lipoic acid synthase deficiency and to assess whether the endogenous deficiency is associated with increased susceptibility to diabetes and cardiovascular diseases. Homozygous Lias deficient mice die in utero, whereas heterozygous deficient mice survive with a decreased antioxidant capacity compared to wild type littermates (Yi *et al*., 2005). These results indicate that lipoic acid synthase is an essential enzyme in normal embryonic development, and synthesis of endogenous LA is also crucial due to its roles as cofactor and antioxidant.

In this study, I have investigated physiological effects of Lias heterozygous deficiency and assessed whether the reduction of endogenous lipoic acid production is associated with the initiation and progression of various forms of human disease under certain pathological conditions. To this end, I administered LPS (lipopolysaccharide), ISO (isoproterenol) and PE (phenylephrine) to Lias heterozygous deficient mice and characterized

genetic effects of Lias heterozygous deficiency upon exogenous oxidative stimulation such as lipopolysaccharide, isoproterenol and phenylephrine..

CHAPTER 2

ASSESSMENT OF LIPOPOLYSACCHARIDE INDUCED PATHOPHYSIOLOGY IN LIPOIC ACID SYNTHASE DEFICIENT MICE

2.1 INTRODUCTION

Oxidative stress or reactive oxygen species (ROS) is defined as cellular damages resulting from an imbalance between an excessive generation of oxidant compounds and insufficient antioxidant defense mechanism (Sies *et al*., 1997). Reactive oxygen species have been extensively studied and evidences indicated that oxidative stress and oxidation process may damage cellular macromolecules and resulting in progression of several chronic diseases (Wollin *et al*., 2003). Currently, it has been observed that complex human diseases such as atherosclerosis and diabetes are associated with increased levels of cellular oxidative stress (Madamanchi *et al*., 2005; Sachidanandam *et al*., 2005, Maritim *et al*., 2003). Up to date, several experimental models and clinical investigations have evaluated benefits of LA/DHLA as a potent antioxidant in prevention and treatment of diabetes and cardiovascular diseases (Wollin *et al*., 2003; Osiecki *et al*., 2004). However, its molecular anti-oxidizing mechanism involved in reduction of naturally occurred oxidative stress and pathophysiological roles associated with complex human diseases have not been clarified in details. Moreover, there is no concrete knowledge describing how the endogenous lipoic acid synthesis is modified under certain pathological conditions and whether the genetic alterations of lipoic acid synthesis will modify the development of diseases (Yi *et al*., 2005).

To identify underlying mechanisms of lipoic acid as an antioxidant and unravel pathophysiological roles of lipoic acid against complex human diseases, we used lipoic acid synthase deficient mice generated by targeting *Lias* gene responsible for synthesis and endogenous production of lipoic acid (Yi *et al*., 2005). Previous experimental results showed that homozygous *Lias* deficient mice die in utero whereas heterozygous survive with increased oxidative stress, reduced antioxidant capacity compared to wild type littermates. These findings suggest that lipoic acid and its synthesis play crucial roles in normal development, energy metabolism and endogenous antioxidizing mechanism (Yi *et al*., 2005), and It can be speculated that Lias deficiency may be more vulnerable to various forms of complex human diseases associated with exogenous oxidative stress.

LPS (lipopolysaccharides) is a large molecular compound composed of lipid and carbohydrate, and is a major protective cellular structure of Gram-negative bacteria (Henderson *et al*., 1996). Commonly, LPS acts as an endotoxin that induces septic shock and cascade of systemic pro-inflammatory reactions triggered by macrophages, cardiovascular failures, internal organ damage and even death (Henderson *et al*., 1996; Lynn *et al*., 1998). In addition, LPS also can increase reactive oxygen species production by generating free radicals which lead to oxidative stress at given species, thus it is a widely accepted and used reagent to induce oxidative stress (Galley *et al*., 1997; Goode *et al*., 1995; Loft *et al*., 1998).

In this study, we tested hypothesis that reduced lipoic acid synthase gene expression increases lipopolysaccharide-induced oxidative stress and tissue damage in lipoic acid synthase heterozygous deficient mice compared to wild type mice. Our data demonstrate that lipoic acid synthase heterozygous deficient mice show an increased recruitment of inflammatory cells to lung and liver and markedly enhanced tissue damage following an LPS

administration, and implicates that Lias deficiency has increased sensitivity upon LPS-induced inflammatory reaction and septic response.

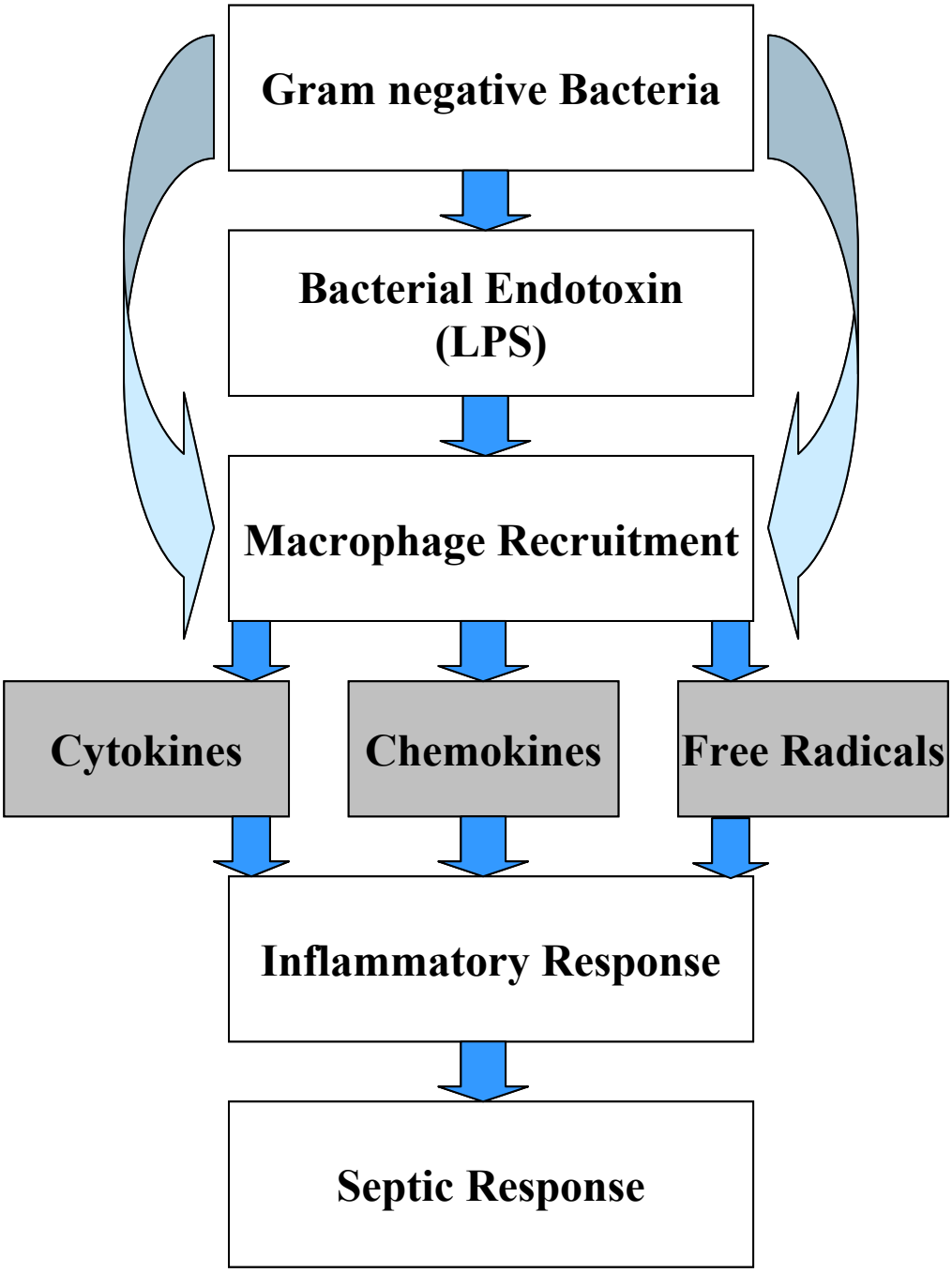

Figure 2.1. The LPS and pathway of sepsis. LPS is produced by the Gram negative bacteria. Upon administration, recruited macrophages generate various cytokines, chemokines and free radicals which induce local inflammation. Prolonged inflammation will result in septic shock and organ damage.

2.2 MATERIALS AND METHODS

2.2.1 Mice and LPS administration

The Lias heterozygous mice and their wild type litter mates were F1 between wild type C57BL/6J mice and 129/SvEv mice heterozygous for the disrupted Lias gene. Experiments were conducted on young (3 months old) female Lias heterozygous and wild type mice. Lipopolysaccharide (Sigma, *E. Coli* 055:B5) of 20 mg/kg of body weight was administered intraperitoneally (I.P.) to conscious mice whereas control mice received PBS. All animals were housed at the room temperature (22-24°C) with free access to laboratory chow and drinking water and were exposed to alternate cycles of 12-hour light and dark cycle until the time of sacrifice. Mice were maintained according to the Guide for Care and Use of Laboratory Animals, published by the NIH in U.S., and all experiments were carried out in accordance with protocols approved by the IACUC of the University of North Carolina at Chapel Hill.

2.2.2 Plasma Metabolites, Body Temperature

Mice were fasted for approximately 4-5 hours before collection. Retro-orbital bleeding was performed with heparinized capillaries to obtain blood from each animal. Collection tubes were anti-coagulated with 0.5M of EDTA and whole blood was then centrifuged at 4°C to separate the plasma. Plasma levels of glucose (WAKO, Richmond, VA), triglyceride (Stanbio, Boernl, TX), and cholesterol (WAKO, Richmond, VA) were determined by enzymatic reaction of 2ul of plasma in 200ul of commercially available solution followed by spectrophotometer quantitation. Rectal temperature sensors (Physitemp,

Physitemp Instruments Inc, Co., Clifton, NJ) was placed and body temperature of each animal was recorded before and every 60 minutes for 4 hour after LPS administration.

2.2.3 Hematological and Blood Assays

Retro-orbitally drawn blood at 0hr, 2hr, 4hr, 8hr and 16hr after LPS injection was analyzed for hematological assays. Leukocyte and platelet counts were measured on an Animal Blood Counter, CBC-Diff™ (Heska Inc. Loveland, CD). LPS-induced liver and kidney damages were assessed by measuring the activities of plasma alanine aminotransferase (ALT), aspartate aminotransferase (AST) and blood urea nitrogen (BUN) using a Vitro 250 Chemical Analyzer (Ortho-Clinical Diagnostics, Inc., Raritan, NJ). Plasma levels of cytokines and chemokines were quantified by commercially available kit which is specific for mouse (Linco Research, St. Charles, MI). Levels of IL-6, IL-10 (Interleukin-10), TNF-α (Tumor necrosis factor-alpha) and MCP-1 (Monocyte chemoattractant protein-1) were measured on the Luminex 100 system and analyzed with the Masterplex QT 1.2 or LMAT software (Luminex Corporation, Austin, TX).

2.2.4 Biochemical Analyses and Tissue Preparation

Liver, lung, heart, kidney, spleen and pancreas were surgically removed and quick frozen in liquid nitrogen and stored at -80°C for subsequent biochemical analyses. Tissues were homogenized in 1ml of ice-cold 50mM potassium phosphate buffer (pH 7.4) and then centrifuged at 4°C of 10,000g for 30 minutes. The supernatants were used for measurement of NOx, GSH and TBARS. The infiltration and activation of neutrophils were determined indirectly by measuring the activity of MPO (myeloperoxidase) in lung and hepatic

homogenates as described (Suzuki *et al*., 1994). Measurement of the antioxidant capacity, GSH (reduced glutathione) level of the erythrocyte and tissue was determined using a commercially available kit (Calbiochem, San Diego, CA) following the manufactures' protocol. Measurement of the lipid peroxidation, TBARS (Thiobarbituric Acid Reactive Substances) assay and NO (Nitric Oxide, Nitrate/Nitrite Oxide concentration) was measured. TBARS assay was performed in the supernatant of homogenized tissue lysate of heart, liver and kidney as previously described (Lapenna *et al*., 2001) using MDA (malondialdehyde) as a standard. For NO assay, plasma levels of Nitrate/Nitrite Oxide concentration was determined at 0hr, 4hr and 8hr after LPS administration by using commercially available kit (Cayman, Ann Arbor, MI) following the manufactures' protocol, and the concentration of nitrite was calculated from a standard curve of $NaNO_2$ (5-35 mmol/L) and expressed as mmol/L of nitrate. Nuclear extract of livers was freshly prepared by using the Nuclear Extraction Kit (Active Motif, Carlsbad, CA) following manufactures' protocol. NF-κβ activation in liver tissues was monitored using a TransAM NF-κβ p65 Transcription Factor Assay Kit (Active Motif, Carlsbad, CA) following manufactures' protocol. Protein concentrations of tissue homogenates were determined based on the Bradford method (Bio-Rad Laboratories, Hercules, CA) and known concentrations of BSA (bovine serum albumin) was used as a standard.

2.2.5 RNA Isolation & Gene Expression Analysis

Tissue from liver, heart, spleen, kidney and pancreas were transferred immediately after surgical removal and stored in RNA later solution (Ambion, Austin, TX) overnight. RNA isolation was done by the ABI 6700 RNA automated robot, approximately 30 to 100mg

of tissue was weighed and was put into 1ml of lysis buffer (ABI, diluted with 1X PBS). Samples were then homogenized thoroughly for 30 seconds for two times. Cooled down for five minutes and stored overnight at 4°C. Roughly, 10 to 20µl of proteinase K (10ug/µl) was added into lysate, incubated at room temperature for an hour and then cooled down at ice for five minutes. Approximately, 200µl out of 1ml of lysate was applied to ABI 6700 RNA extraction robot. Remaining steps were followed by the manufactures' protocol. Levels and patterns of mRNA expression of *Lias, MnSOD, TNF-α, ICAM, VCAM, E-Selectin, L-Selectin, GRO, MIP1-α, MCP1, eNOS, iNOS, IFN-γ, NF-κβ* were determined by TaqMan real-time quantitative reverse transcription PCR (RT-PCR), with β-actin as the reference gene in each reaction (Kim *et al*., 2002). Sequences of all TaqMan primers and probes are summarized in Table 2.1.

2.2.6 Histological Analysis and Immunofluorescence

Liver, heart, kidney, spleen and pancreas tissue samples were surgically removed and immediately kept in 4% PFA (pH 7.4) solution, and then fixed overnight. Samples were trimmed and sent to the Histological Core Facility at University of North Carolina at Chapel Hill for further process. Five micron sections were stained with H&E (hematoxylin and eosin) and Mason's trichrome staining. Lipoic acid in tissue sections were visualized by the immunoflourescence procedures with a rabbit anti-lipoic acid polyclonal antibody (Calbiochem, San Diego, CA) and a goat anti-rabbit IgG antibody conjugated with Alexa Fluor 594 (Invitrogen, Carlsbad, CA). Slides were examined using a Nikon Eclipse TE300 microscope attached with either a Nikon D100 camera or a CoolSnap Photometrics HQ camera from Media Cybernetics with Image-Pro Plus 5.0 software.

2.2.7 Statistical Analysis

Statistical analyses were carried out with JMP software (SAS, Cary, NC). Values reported here is mean ± SEM. Effect of genotype and drug treatment was analyzed by two-factorial ANOVA followed by Tukey-Kramer test. Student's *t* test was used for comparison between heterozygous and wild type mice group. $P<0.05$ was considered as a statistical significance.

2.3 RESULTS

2.3.1 Energy Metabolism in Lias Heterozygous deficient mice after LPS administration

Since lipoic acid is an important cofactor for the enzyme complexes locating in the TCA cycle and participating in oxidative metabolism of glucose, blood glucose and body temperature at different time points following LPS administration were monitored in the Lias heterozygous mice (n=10) and wild type mice (n=12). Similar as septic shock patients, the LPS-administered mice showed a typical biphasic response of hepatic carbohydrate metabolism (Maitra *et al*., 1999) with an initial hyperglycemia followed by a profound hypoglycemic phase (Fig 2.2.). No significant difference was observed in blood glucose levels at any time point between Lias heterozygous and wild type mice, however, the blood glucose levels in the Lias heterozygous mice tended to be higher at basal level and during hyperglycemic phase but lower during the hypoglycemic phase resulting in a steeper drop between 1 hour to 2 hour after treatment of LPS than wild type mice group (P<0.0012). Marked change in body temperature, either fever or hypothermia, is a hallmark of endotoxemia in laboratory animal experiments (Kozak *et al*., 1994), and this was observed in all LPS-administered mice in our study with the body temperature reduction to mean of 28°C (Fig 2.3.). These data suggest that decreased energy metabolism in the wild type mice upon LPS treatment is the same in the mice with reduced Lias gene activity.

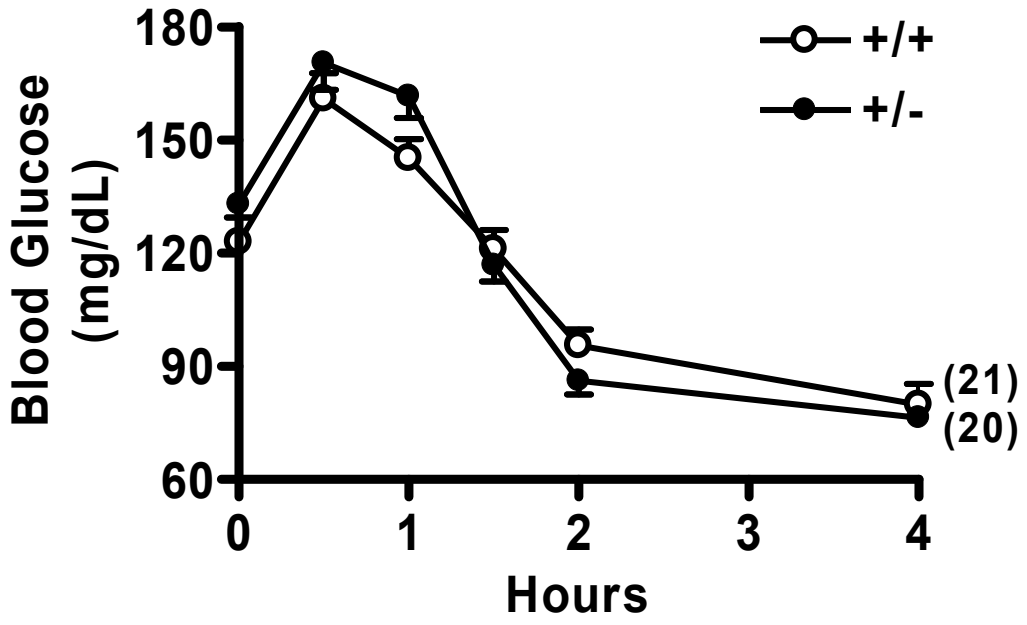

Figure 2.2. Measurement of blood glucose level before and after LPS administration. Blood glucose level was measured before and after the LPS administration in Lias heterozygous and wild type groups. Biphasic response (Hyperglycemia and hypoglycemia) was observed after LPS administration in both genotypes; However, both genotypes developed hypoglycemia.

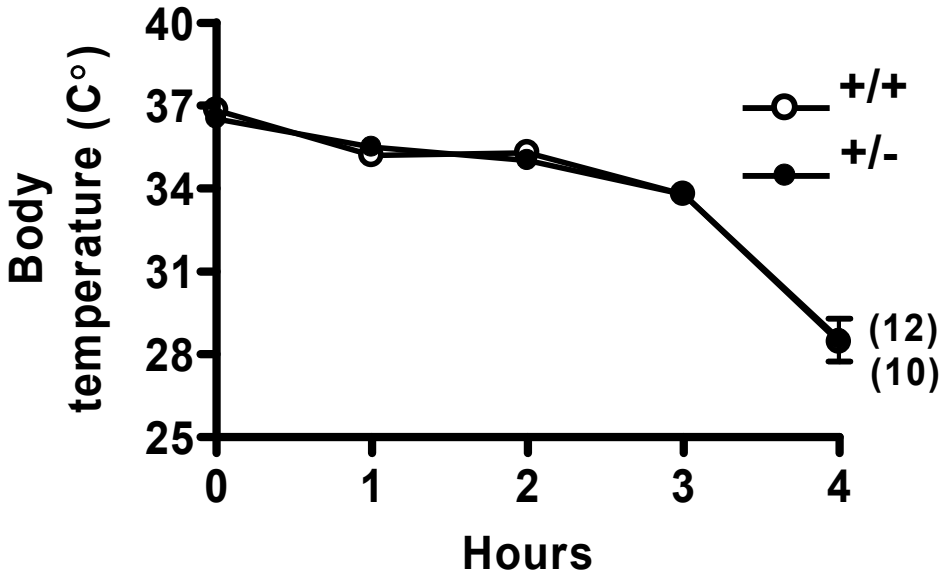

Figure 2.3. The measurement of body temperature before and after LPS administration. The body temperature was measured before and after LPS administration in both genotypes. Both genotypes showed decreased body temperature (hypothermia) after LPS administration, however, genotypic difference was not observed.

2.3.2 Peripheral WBC and Platelet counts in Lias heterozygous deficient mice after LPS administration

The platelet counts fell following injection of LPS similarly in mice of both genotypes until at 4 hour when response of the two genotypes diverged. At 8 hour time point, platelet counts in Lias heterozygous mice continued to reduce whereas wild type group started to recover, however, the counts of the platelet of these two groups reached to similar levels at 16 hour time point (Fig 2.4.). Mean platelet volume in these two groups did not change during this course (Fig 2.5.) implying that decreased platelet numbers in Lias heterozygous mice was not due to the reduction of platelet generation in bone marrow. Likewise, circulating WBC was markedly decreased, reaching to a minimum (Approximately 20%) at 4 hour post-injection of LPS and followed by a return back to an about 50% normal level at 8 hour in wild type mice group. In contrast, Lias heterozygous mice showed a similar degree of reduction but a significant delay in the recovery at 8 hour and 16 hour compared to wild type mice (Fig 2.6.).

To test whether the decreased levels of WBC and platelets were due to enhanced sequestration in tissues in lungs and liver upon LPS challenged Lias heterozygous animals, we measured the activity of MPO, an enzyme primarily produced by neutrophils in lung and liver at 8 hour after LPS challenge. As shown in Fig 2.7., MPO activities in the Lias heterozygous mice were twice as much as that of wild type tissues (Lung:1.13±0.18 U/mg protein vs 0.49±0.06 U/mg protein, $P<0.05$; Liver:0.565±0.1 U/mg protein vs 0.232±0.059 U/mg protein $P<0.05$). These data suggest that lungs and livers of Lias heterozygous mice contain more WBC at 8 hour and 16 hour and that the release of marginated leukocytes to circulation is significantly impaired in Lias heterozygous mice compared to wild type mice.

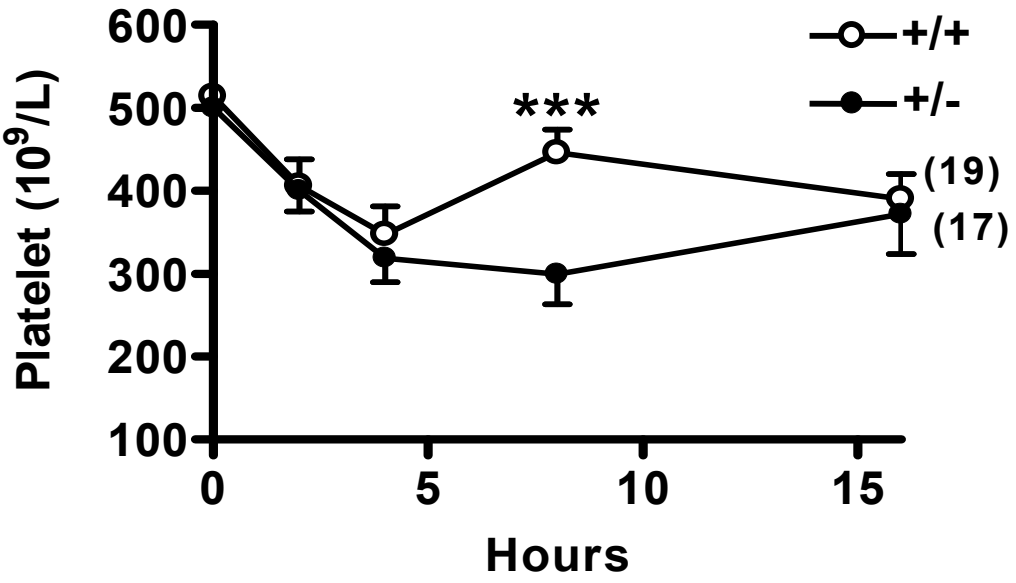

Figure 2.4. Measurement of platelet counts before and after LPS administration. Platelet counts were measured before and after LPS administration. Four hours after LPS administration, Lias heterozygous mice show low counts of platelet and delayed recovery of platelet compared to wild type mice. Both reached similar platelet counts after 16 hours.

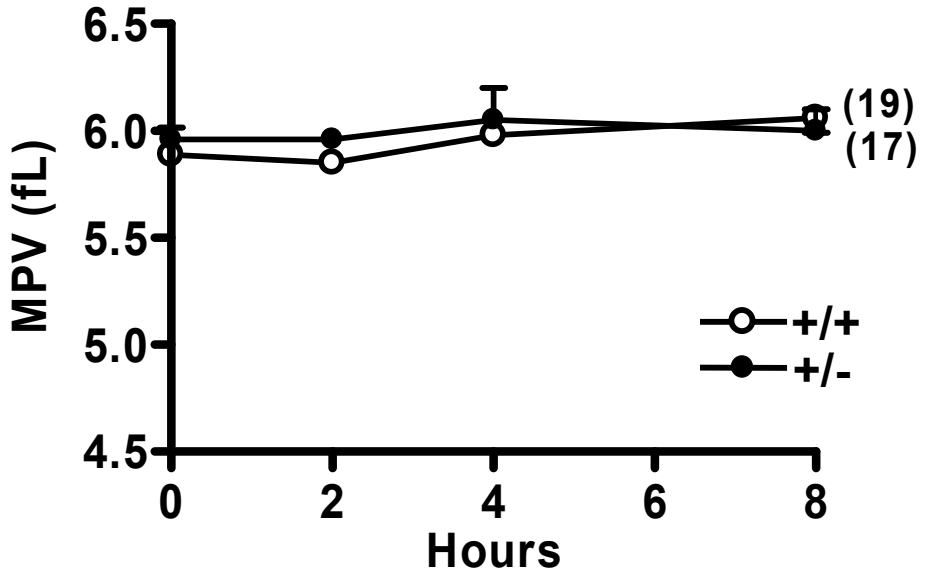

Figure 2.5. Measurement of mean platelet volume before and after LPS administration. Mean platelet volume was measured before and after LPS administration. Their average platelet volume did not change before and after LPS administration, and genotypic difference was not observed.

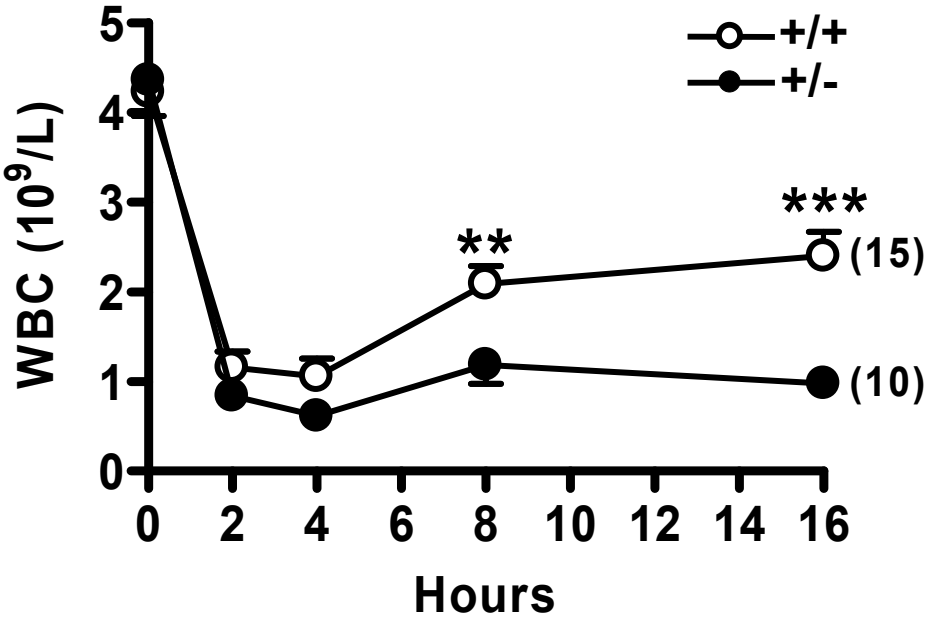

Figure 2.6. Measurement of white blood cell counts before and after LPS administration. WBC counts before and after LPS administration was measured. Both showed decreased WBC after LPS administration, however, Lias heterozygous mice showed significant reduction in WBC counts and delayed recovery compared to wild type littermates.

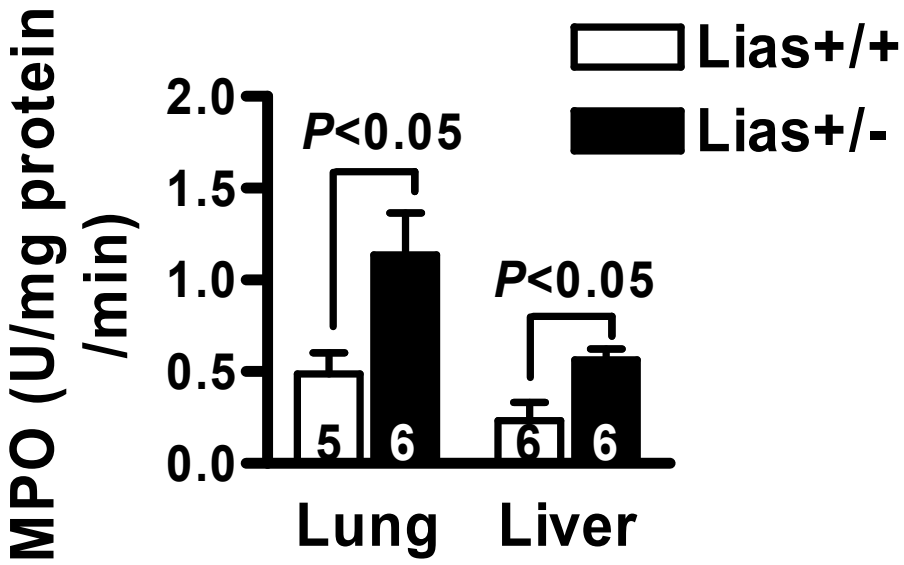

Figure 2.7. Measurement of MPO activity in lung and liver. The levels of MPO (myeloperoxidase) in llung and liver after LPS administration. The MPO activity of the Lias heterozygous mice in lung and liver significantly increased upon LPS administration due to increased levels of neutrophil activity.

2.3.3 Organ damage in Lias heterozygous deficient mice after LPS administration

Leukocytes sequestration in the tissues is thought to be responsible for the organ damage induced by LPS (Lukaszewicz *et al*.,1996). Serum levels of ALT, AST and BUN in the Lias heterozygous and wild type mice were not different in the basal. However, mean serum levels of AST was much higher in the Lias heterozygous mice group than the wild type mice (Lias +/-: 412 U/L, Lias +/+: 178 U/L, $P<0.001$, Fig 2.8.), and the ALT level was also significantly elevated in the Lias heterozygous mice compared to wild type mice group (Lias +/-: 100 U/L, Lias +/+: 68 U/L, $P<0.01$, Fig 2.9.). These data suggest that Lias heterozygous mice are much more sensitive to LPS-induced hepatic damage. Similarly, serum BUN levels in Lias heterozygous mice and wild type were the same before LPS challenge (Lias +/-: 10.4±1.3 mg/dl, Lias +/+: 11.5±1.7mg/dl), but marked increase was detected in both Lias heterozygous and wild type mice after LPS challenge (Lias +/-: 41.6±5.7mg/dl, Lias +/+: 36.4±2.3mg/dl, $P=0.25$, Fig 2.10.). The genotypic effect on serum BUN levels was not significant suggesting that the reduced Lias had a lesser effect on the kidney damage than on the liver damage at the 8 hour time point.

Light microscopic evaluation of the lung and liver in both genotypes at 8 hour after LPS challenge confirmed that the infiltration of inflammatory cells are more extent in the Lias heterozygous mice group than in the wild type mice (Fig 2.11.). Neutrophil infiltration in the lung of the Lias heterozygous mice was also more evident compared to that of wild type mice group. One Lias heterozygous mouse out of total 17 mice had severe liver necrosis while one wild type mouse out of total 16 mice had small but histologically detectable lover necrosis. These observations indicate that the delayed recovery of platelets and leukocyte

counts in circulation of Lias heterozygous mice is associated with enhanced tissue damage in response to LPS challenge.

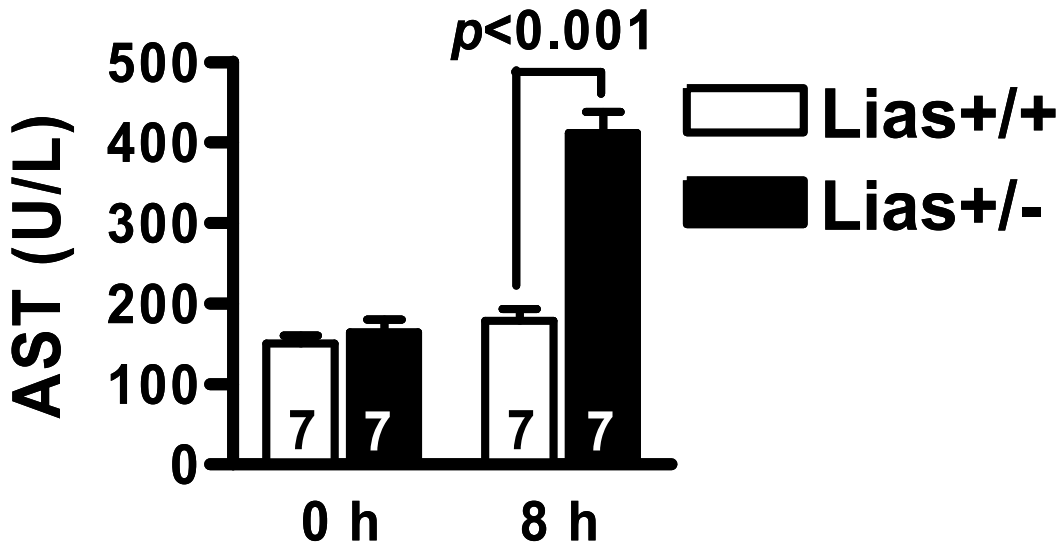

Figure 2.8. Measurements of plasma AST (aspartate aminotransferase) before and after LPS administration. Plasma levels of AST (an indicator of liver damage) were measured before and after LPS administration. Before LPS administration, both genotypes showed no sign of liver damage, however, AST level significantly increased in the Lias heterozygous mice compared to wild type after eight hours of LPS administration. (Black bar represents Lias heterozygous mice and solid bar represents wild type mice, number represents the number of animal used for experiment).

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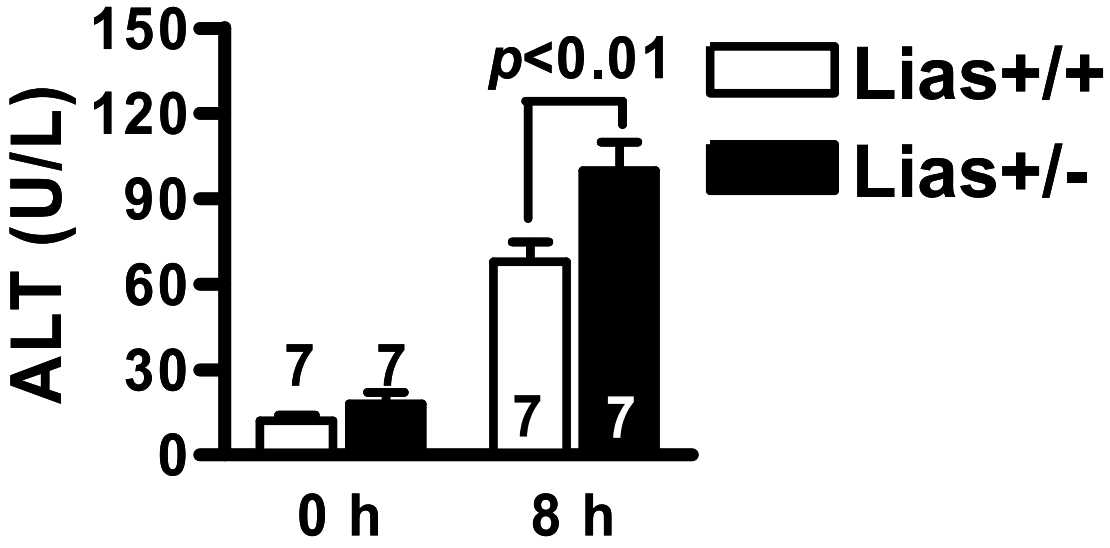


Figure 2.9. Measurement of plasma ALT (alanine aminotransferase) before and after LPS administration. Plasma levels of ALT (an indicator of liver damage) were measured before and after LPS administration. Before LPS administration, both genotypes showed no sign of liver damage, however, ALT level significantly increased in the Lias heterozygous mice compared to wild type after eight hours of LPS administration. (Black bar represents Lias heterozygous mice and solid bar represents wild type mice, number represents the number of animal used for experiment).

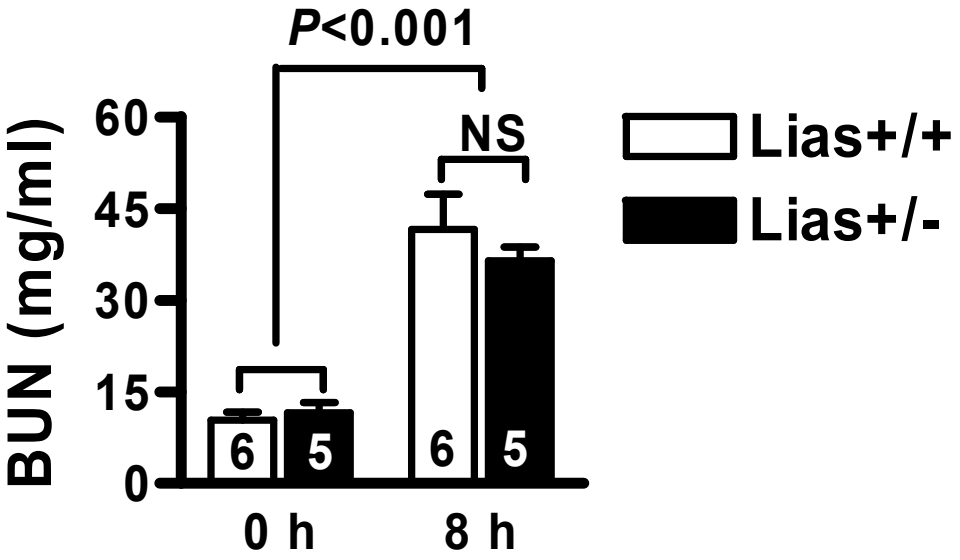


Figure 2.10. Measurement of plasma BUN (Blood urea nitrogen) before and after LPS administration. Plasma levels of BUN (indicator of kidney damage) were measured before and after LPS administration. Before LPS administration, both genotypes showed no sign of kidney damage, however, the levels of BUN significantly increased in both groups but no genotypic difference was observed after LPS administration.

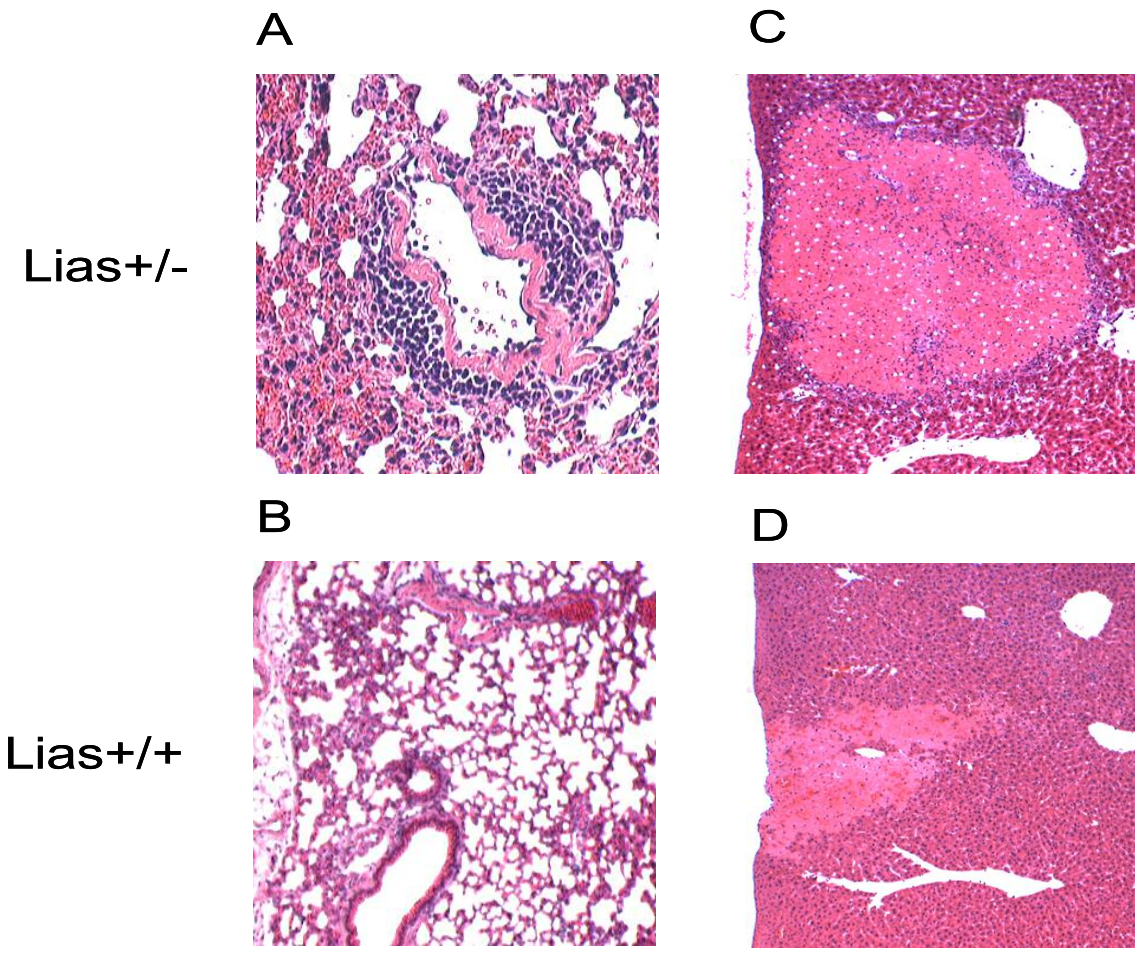


Figure 2.11. Histological analysis of lung and liver after LPS administration. H&E staining of lung and liver tissue. After LPS administration, Lias heterozygous mice showed enhanced infiltration of inflammatory cells and tissue damage in liver and lung compared to wild type littermates.
A, B, lung sections of both genotypes after LPS administration; C, D, liver sections of both genotypes after LPS administration

2.3.4 Induction of Plasma Cytokines

To examine whether the increased tissue damage is related to enhanced inflammatory response to LPS challenge in the Lias heterozygous mice, serum levels of the pro-inflammatory cytokines, TNF-α, IL-6, MCP-1 and the anti-inflammatory cytokine IL-10 were measured at 0, 2, 4, and 8 hour after LPS injection. Basal level of TNF-α, IL-6, IL-10 and MCP-1 in sera of Lias heterozygous mice and wild type mice group was not different. At 2 hour after LPS administration, plasma cytokine levels were significantly increased compared to basal level. The mean serum TNF-α level at 2 hour in Lias heterozygous mice was significantly higher than in wild type mice (Lias +/-: 7.4±0.5 ng/ml, Lias +/+: 4.3±1.0ng/ml, $P<0.01$ Fig 2.12.). IL-10 was also higher in the Lias heterozygous mice at 2 hour, but the difference was not statistically different (Fig 2.13.). Both TNF-α and IL-10 levels were decreased with the time and at 8 hour time point, but the serum levels in both genotypes were not different. In contrast, IL-6 and MCP-1 levels were increased at 2 hours after LPS injection and remained relatively high at 8 hours in mice with both genotypes (Fig 2.14., Fig 2.15.). These data indicate that the inflammatory cytokine response in the mice with heterozygous deficiency of the Lias may be exaggerated in the very early phase of infection, but not in the later phase of infection.

NF-κβ regulates inflammatory reactions through its binding to the promoter regions of many genes including TNF-α. To investigate whether the sensitivity of Lias heterozygous mice to LPS challenge occurs via the activation of NF-κβ signaling pathway, NF-κβ DNA-binding activity was examined in the nuclear extracts of liver tissue from the untreated and LPS-administered mice. NF-κβ p65 was highly activated in the livers of mice of the both genotypes at 8 hour after LPS challenge as demonstrated by the five fold increase in the

absorption, at the OD_{450nm} (0.49±0.1 vs 0.1±0.01, $P<0.001$, Fig 2.16.). However, levels of NF-κβ activation in Lias heterozygous and wild type mice were virtually were identical, suggesting that the activation of NF-κβ is not responsible for the increased tissue damage seen in the Lias heterozygous mice group at 8 hour post infection.

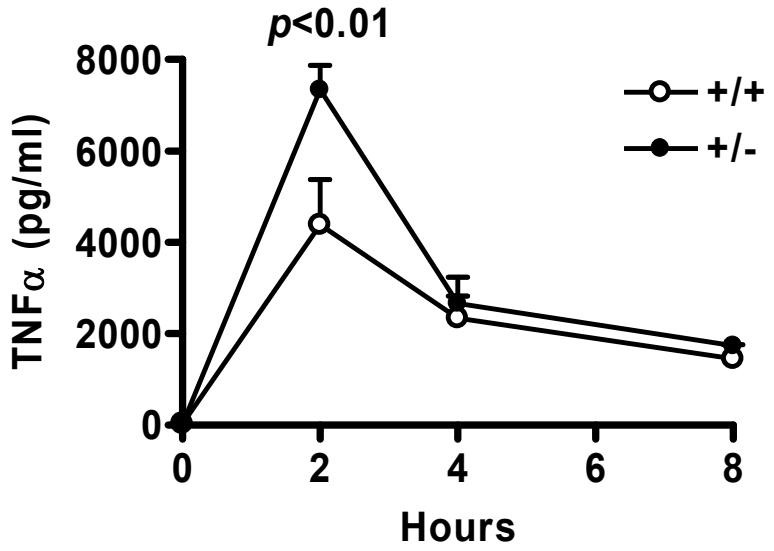


Figure 2.12. Measurement of plasma TNF-alpha before and after LPS administration. The plasma levels of TNF-alpha significantly increased after 2 hour of LPS administration in Lias heterozygous mice compared to wild type littermates, however, both genotypes reached similar levels after 4 hour of LPS administration.

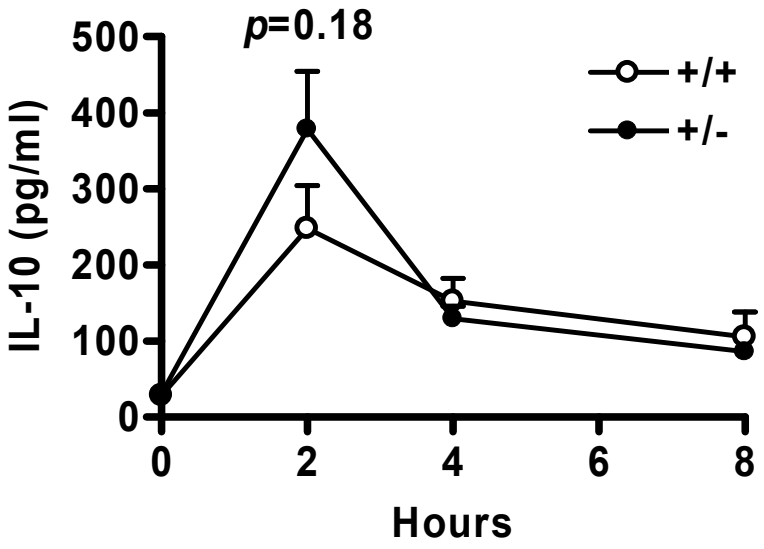


Figure 2.13. Measurement of IL-10 before and after LPS administration. The plasma levels of IL-10 increased after 2 hour of LPS administration in Lias heterozygous mice compared to wild type littermates but did not reach statistical significance. However, both genotypes reached similar levels after 4 hour of LPS administration.

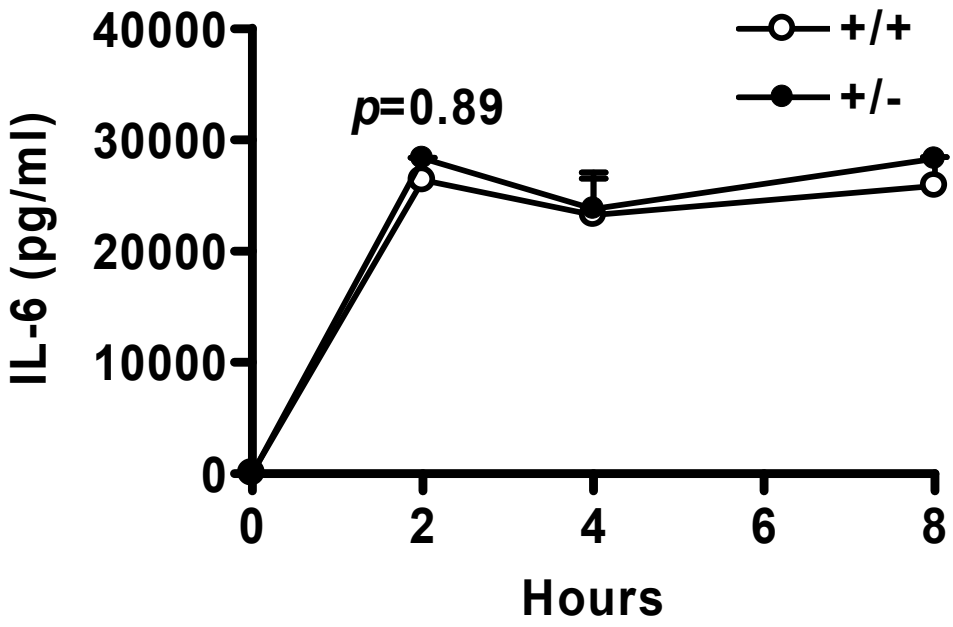


Figure 2.14. Measurement of plasma IL-6 before and after LPS administration. The plasma levels of IL-6 increased after 2 hour of LPS administration in groups of mice, however no genotypic difference was observed.

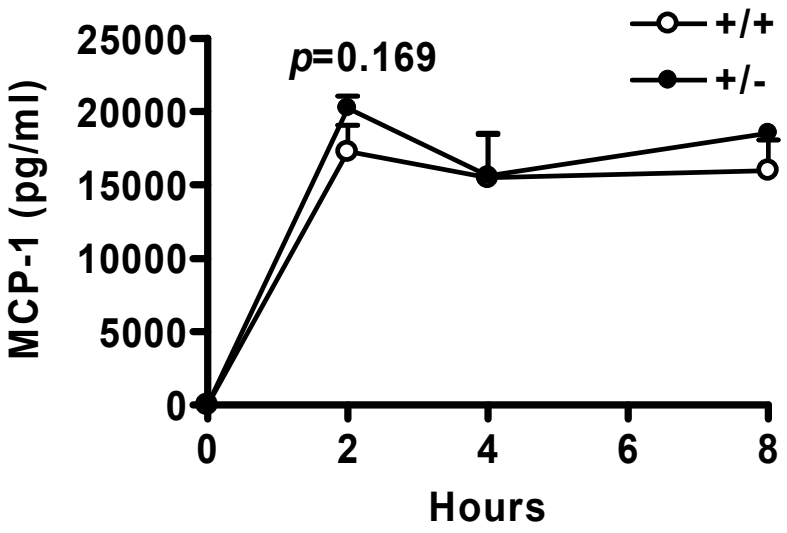


Figure 2.15. Measurement of plasma MCP1 before and after LPS administration. After LPS administration, plasma levels of MCP1 increased in both genotypes and remained relatively high during the experiment. No genotypic difference was observed.

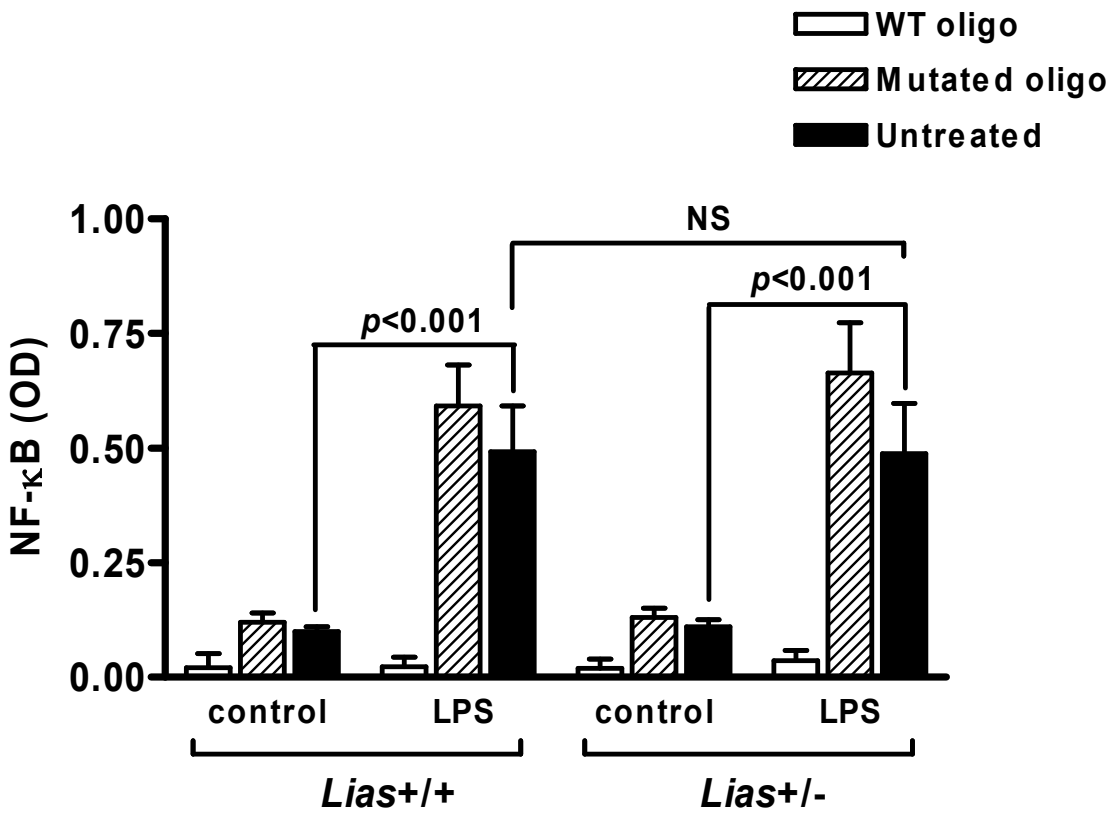


Figure 2.16. Measurement of NF-κβ in liver before and after LPS administration. After LPS administration, NF-κβ activation was significantly increased in both genotypes. However, genotypic difference was not observed in NF-κβ activity upon LPS administration.

2.3.5 Increased Oxidative Stress in Lung and Liver

LPS-induced septic shock increases oxidative stress (Galley *et al*., 1997; Goode *et al*., 1995; Hewett *et al*., 1993; Macdonald *et al*., 2003; Shenkar *et al*., 1999; Sakaguchi *et al*., 2006; Takeda *et al*., 1986). We, therefore, examined GSH in erythrocyte, lung and liver, and TBARS in plasma, lung and liver at 8 hour after LPS-injection. Plasma TBARS was increased and erythrocyte GSH was decreased by LPS challenge, but there was no statistically significant difference between two genotypes. In contrast, TBARS in lungs and livers of Lias heterozygous mice at 8 hour post infection were 70% and 100% more respectively, than those in the wild type mice group while liver GSH was significantly lower (Fig 2.17., Fig 2.18.). Similarly, serum nitrite levels were increased 10 times in response to LPS challenge in the both wild type (211.2±20.9μM at 8 hour vs. 20±2.1μM basal level, $P<0.001$), but not statistically significant (Fig 2.19.). The Lias heterozygous mice had slightly higher NOx levels in the liver than wild type mice (Lias +/-: 0.91±0.19μmol/mg protein, Lias +/+:0.81±0.10μmol/mg protein, $P=0.6$), but the difference between two genotypes was not significant.

As expected, the basal expression of the Lias gene assessed by the real time RT-PCR in the livers of the Lias heterozygous mice were approximately 50% of that in the wild type mice. At 8 hour after LPS-injection, the Lias gene expression was markedly reduced by in both groups by 70% ($P<0.001$, Fig 2.20.). Immunostaining of the liver sections with anti-LA antibody showed that the number of LA-positive hepatocytes of the Lias heterozygous mice was much less than wild type mice group after LPS-challenge (Fig 2.21) suggesting that, in addition to the reduced lipoic acid production, lipoic acid in the liver of Lias heterozygous mice was consumed to a greater extent. These results suggest that although the general

oxidative stress in response to LPS treatment as measured in the plasma is the same, the tissue of the Lias heterozygous mice demonstrate increased oxidative stress than those of wild type mice, reflecting an impaired endogenous antioxidant reserve and/or increased damage in these tissues.

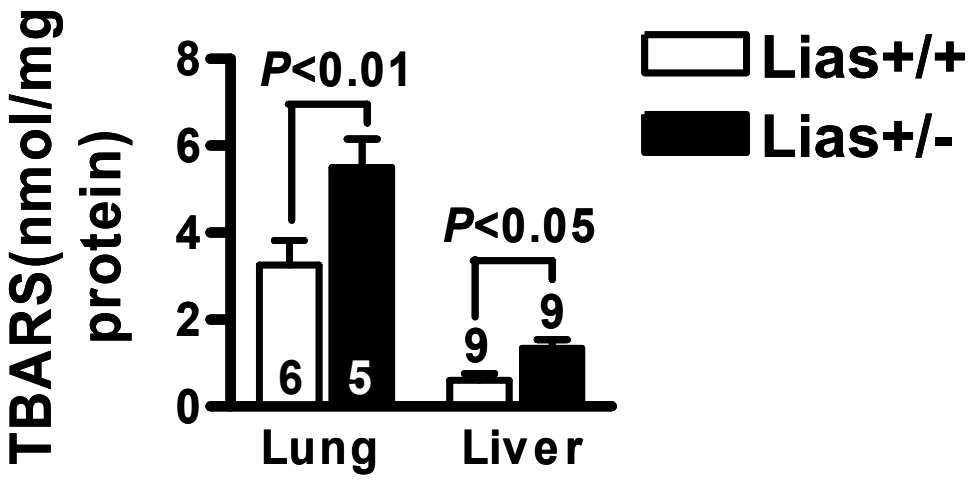


Figure 2.17. Measurement of TBARS in lung and liver after LPS administration. After LPS administration, levels of lipid peroxidation, TBARS, significantly increased in lung and liver of Lias heterozygous mice compared to wild type littermates. (Black bar represents Lias heterozygous mice and solid bar represents wild type mice, number represents the number of animal used for experiment).

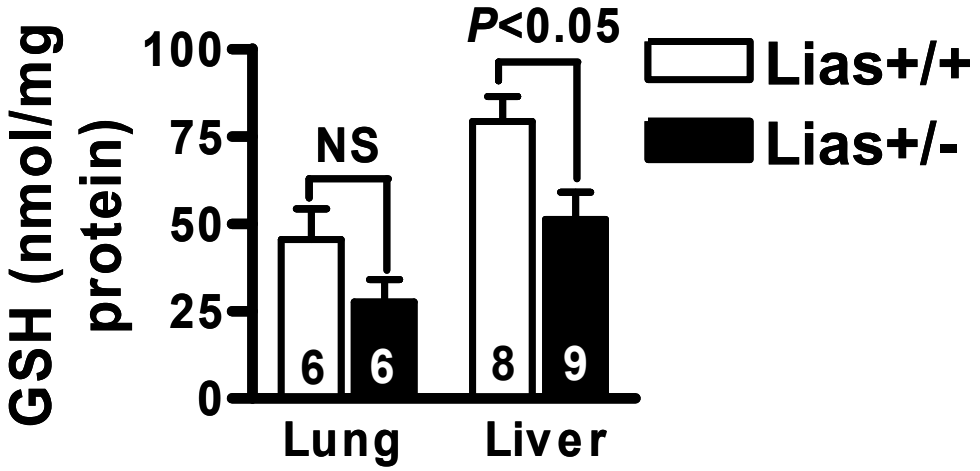


Figure 2.18. Measurement of GSH in lung and liver after LPS administration. After LPS administration, levels of antioxidant capacity, GSH (reduced glutathione), significantly decreased in liver and lung of Lias heterozygous mice compared to wild type littermates. (Black bar represents Lias heterozygous mice and solid bar represents wild type mice, number represents the number of animal used for experiment).

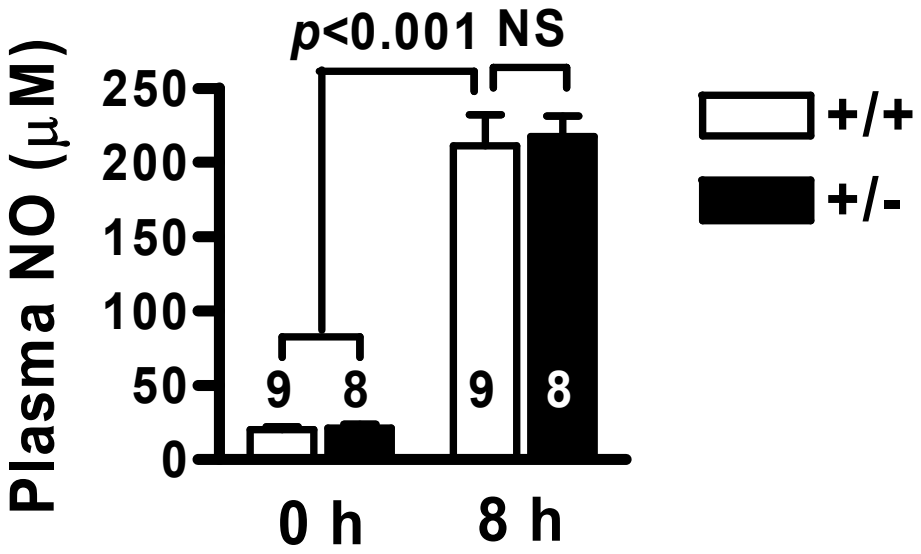


Figure 2.19. Measurement of plasma NO before and after LPS administration. Before LPS administration, plasma levels of NO (indicator of oxidative stress) shows relatively low, however, both genotypes significantly increased after LPS administration. No genotypic difference was observed upon LPS administration.

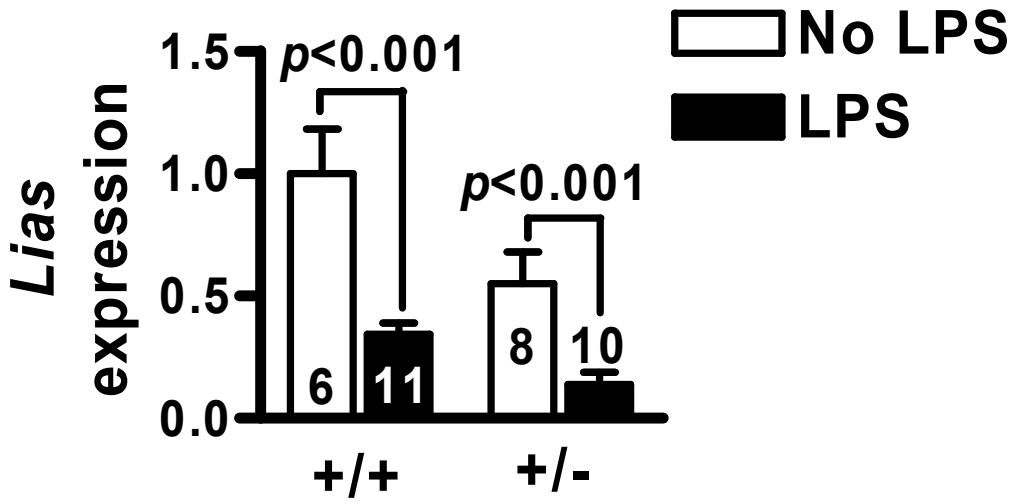


Figure 2.20. Measurement of lias gene activity in liver before and after LPS administration. As expected, Lias heterozygous mice showed half reduced levels of Lias gene in the liver. After LPS administration, lias gene expression was significantly decreased in both genotypes, however, genotypic difference was not observed.

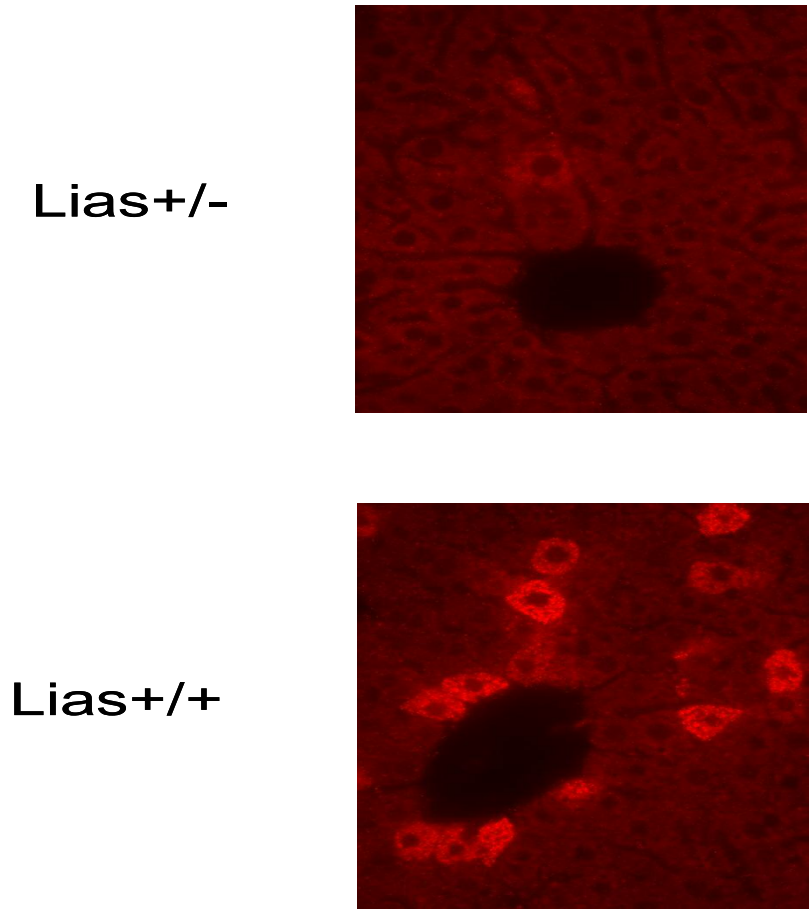


Figure 2.21. Immunoflourescence of lipoic acid in liver after LPS administration. The level of lipoic acid upon LPS administration is decreased more in Lias heterozygous mice compared to wild type littermates, and this indicates that lipoic acid is consumed more in Lias heterozygous mice compared to wild type mice.

2.3.6 Enhanced Expression of Inflammatory Genes

To gain further insights into the mechanisms underlying the enhanced tissue sequestration of leukocytes in the Lias heterozygous mice compared to wild type mice, we analyzed the expression of some of the LPS-inducible genes in the livers at 8 hour post infection by real time RT-PCR (Table 2.2.). Among cytokines and chemokines important for recruiting white blood cells into the tissues, expression in the Lias heterozygous livers of *GRO* gene, a major chemoattractant protein for neutrophils, was three fold that in the wild type liver, while those for *MIP1-α* and *MCP1*, two major chemoattractant proteins for macrophages, were not significantly different between two groups. The expression trend of *TNF-α* was higher in Lias heterozygous mice than wild type mice. Of the genes related to leukocyte trafficking, small increases were seen in intracellular cell adhesion molecule (*ICAM-1*) and vascular cell adhesion molecule (*VCAM-1*) expression in the Lias heterozygous mice livers, but expressions of *L-selectin* and *E-selectin* were not different. Lias genotype had no effects on the expression of the genes for *MnSOD*, *iNOS* and *CD14* in the liver. Taken together, these gene expression data suggest that the impaired recovery of the plasma WBC and platelet counts after LPS-administration in the Lias heterozygous mice is likely due to enhanced tissue response leading to continual recruitments of neutrophils via GRO and infiltration through increased expression of cell adhesion molecules on endothelium.

Table 2.1.
TaqMan Primers and Probes

Gene	Type	Sequence (5'-3')
Lias	Forward	GGG TCT GGA TTA TGT TGT CC
	Reverse	CAT GAC ACG GTC TTG GCG AT
	Probe	f CCC GTC GGC CAC ATC ATC TCG AT q
MnSOD	Forward	AAG GAG CAA GGT CGC TTA CA
	Reverse	AGC AGC GGA ATA AGG CCT GT
	Probe	f TGC TGC CTG CTC TAA TCA GGA CCC A q
TNF-a	Forward	CAC ACT CAG ATC ATC TTC TCA A
	Reverse	AGC TGC TCC TCC ACT TGG T
	Probe	f AGC CTG TAG CCC ACG TCG TAG CA q
ICAM-1	Forward	ACT GAG GAG TTC GAC AGA AC
	Reverse	AGG ACC GGA GCT GAA AAG TT
	Probe	f CCG CTA GCT CCA AAA CGC AGC G q
VCAM-1	Forward	GTA TCC ACG TGG ACA TCT AC
	Reverse	CAC TTG ACT GTG ACC GGC TT
	Probe	f TCA ATT CAG TGG CCC CCT GGA GG q
E-Selectin	Forward	GGA TGC CGC CTA CCT GTG AA
	Reverse	AGG TTC CTG CCG CAG AAA GT
	Probe	f TCA GCC CCA CCC GTC CCT TGG TA q
L-Selectin	Forward	AAC ACA GTG TGG AGC ATC TG
	Reverse	CTC CAA AGG CTC ACA CTG GA
	Probe	f TGG TCA TCT CCA GAG CCA ATC TGC CA q
GRO	Forward	GCT TGA AGG TGT TGC CCT C
	Reverse	AGG CAA GCC TCG CGA CCA T
	Probe	f ACC CAA ACC GAA GTC ATA GCC ACA CTC q
MIP-1a	Forward	ACC AAG TCT TCT CAG CGC CA
	Reverse	AAT CTT CCG GCT GTA GGA GA
	Probe	f AGC TGA CAC CCC GAC TGC CTG CTG q
MCP1	Forward	CTG GAG CAT CCA CGT GTT G
	Reverse	TGG GAT CAT CTT GCT GGT GA
	Probe	f AGC CAG ATG CAG TTA ACG CCC CAC T q
eNOS	Forward	GAC AGA CTA CAC GAC ATT GAG
	Reverse	ATG GTC CAG TTG GGA GCA TC
	Probe	f CTA CAA CCT GCC CCC ATG ACT TTG q

iNOS	Forward	GAG CCT TTA GAC CTC AAC AG
	Reverse	TGC TGG GAT TTC AGC CTC AT
	Probe	f CCT CAG CAG CAT CCA TGC AAA GAA CG q
IFN-γ	Forward	GTG GCA TAG ATG TGG AAG AAA AGA
	Reverse	TTT CAT GTC ACC ATC CTT TTG C
	Probe	f TC TCT TCT TGG ATA TCT GGA GGA ACT q
NF-κβ	Forward	AAG TGA TCC AGG CAG CCT TC
	Reverse	CTA TGT GCT GCC TCG TGG AG
	Probe	f TC AGG TCC ACT GTC TGC CTC TCT CGT q
MCAD	Forward	GAG CAG GTT TCA AGA TCG CA
	Reverse	ACT TCG TGG CTT CGT CTA GA
	Probe	f CTA GCC CGA CAG CGC CAG CT q
β-MHC	Forward	AGC TCA GCC ATG CCA ACC GT
	Reverse	TGA GTG TCC TTC AGC AGA CT
	Probe	f AGG CTC TTC ACT TGT TTC TGG GCC TCA q
ANF	Forward	GAG AAG ATG CCG GTA GAA GA
	Reverse	AAG CAC TGC CGT CTC TCA GA
	Probe	f ATG CCC CCG CAG GCC CGG q
Collagen I	Forward	AGA GCA TGA CCG ATG GAT TC
	Reverse	ATT AGG CGC AGG AAG GTC AG
	Probe	f CTC CGA CCC CGC CGA TGT CG q
Collagen III	Forward	CAG TTC TAG AGG ATG GCT GT
	Reverse	TAG TCT CAT TGC CTT GCG TG
	Probe	f CTG TCT TGC TCC ATT CCC CAG TGT G q
β-Actin	Forward	CTG CCT GAC GGC CAG GTC
	Reverse	CAA GAA GGA AGG CTG GAA AAG A
	Probe	t CAC TAT TGG CAA CGA GCG GTT CCG q

f, Reporter dye1 (FAM:6-carboxyfluorescein); t, Reporter dye2 (TET:Tetrachloro-6-carboxyfluorescein); q, Quencher dye (TAMRA: 6-carboxytetramethyl1-rhodamine)

Table 2.1. RT-PCR sequences. Sequences of forward, reverse primers and probes of all mRNA analysis.

Table 2.2. Relative gene expression in the liver of mice at 8hr after LPS infection

Gene	Lias+/+	Lias+/-	P
GRO	0.82±0.24 (6)	2.35±0.46 (6)	0.02
MIP1α	1.27±0.25 (6)	2.20±1.22 (6)	0.47
MCP1	1.13±0.25 (6)	1.00±0.21 (5)	0.71
TNFα	1.28±0.29 (6)	1.92±0.22 (5)	0.12
ICAM-1	0.90±0.11 (12)	2.72±0.96 (11)	0.06
VCAM-1	1.00±0.16 (12)	1.73±0.26 (11)	0.03
L-selectin	1.04±0.11 (6)	1.25±0.11 (5)	0.21
E-selectin	1.18±0.30 (6)	1.13±0.20 (5)	0.91
MnSOD	0.95±0.09 (12)	1.04±0.13 (11)	0.57
iNOS	1.49±3.5 (9)	1.95±0.46 (8)	0.44
CD14	1.06±25 (9)	1.03±0.14 (8)	0.93
PaiI	1.06±0.15 (6)	0.75±0.19 (5)	0.22

Table 2.2. Relative gene expression in the liver of mice at 8hr after LPS infection. Data are expressed as mean ±SE relative to the mean expression in the Lias+/+ liver as 1.00. The number of animals is in parentheses. P values between the two genotypes were calculated by Student's t-test.

2.4 DISCUSSION

In this study, we found that the Lias heterozygous mice are more sensitive to LPS induced oxidative stress and tissue damage. They develop thrombocytopenia and leucopenia in early phase of infection to similar degrees with wild type mice, but are significantly impaired in their recovery at 8 hour post infection. Higher expression of genes for *GRO* and *VCAM-1* and diminished lipoic acid in the Lias heterozygous mice liver compared to wild type suggest that reduced ability to combat with oxidative stress caused continual recruitment and infiltration of neutrophils leading to an exacerbated tissue damage.

LPS is known to induce inflammatory reactions and oxidative stress in experimental animals. Thrombocytopenia, leukocytosis and hyperglycemia followed by hypoglycemia are among the hallmarks of early endotoxinemia, and a marked increases of serum cytokines, such as TNF-α and IL-1, have been implicated for the cause of these symptoms. We found that TNF-α, a pro-inflammtory cytokine, and IL-10, an anti-inflammatory cytokine were higher in Lias heterozygous mice than in wild type mice at 2 hour after LPS challenge. However, their levels at later stages and those of IL-1b and MCP-1 did not differ between the two genotypes, although they were markedly increased over the basal levels. NF-κβ regulates many gene expression such as TNF-α through its binding sites in the promoter region (Collins *et al*., 1995) and the NF-κβ activity in the liver increased about 4-5 fold at 8 hour upon LPS challenge. Nevertheless, since almost the same amount of NF-κβ was activated in both groups, NF-κβ activation may not be the major cause of the enhanced susceptibility to the LPS induced tissue damage of the mice with heterozygous deficiency of *Lias*. Similarly, although some evidence suggests that continual production of NO derived from up-regulation of NO synthase after LPS challenge may cause hepatocellular damage, either directly or

indirectly by forming reactive nitrogen intermediates (Li *et al*., 1999), we observed no significant differences in the NO levels in both plasma and liver between the two genotypes. Likewise, upregulation of iNOS gene expression induced by LPS were same in both genotypes. These findings differ from the effects of exogenously administered LA observed by other investigators. For example, Suntres reported that intraperitoneal pretreatment with LA significantly alleviated LPS induced tissue injuries by decreasing LPS induced TNF-α and NO concentration in plasma and lipid peroxidation in liver (Suntres, 2003). Similarly, it has been reported that LA inhibits the activation of NF-κβ in human T cells exposed TNF-α (Roy *et al*., 1998) and in cultured aortic endothelial cells challenged with advanced glycation end products (Bierhaus *et al*., 1997).

Our results showed that the neutrophil, lymphocyte and monocyte counts continuously drop during the first 4 hours of infection, but started to recover after 4 hours in wild type mice. The initial drop could be explained by destruction of peripheral blood cells, shift of the cells from circulating pool to the marginated pool and/or hemorrhage and sequestration within the tissue. The subsequent rise of blood leukocyte counts in these mice may be explained by cellular release from the marrow reservoir, or a reversal of the shift from a marginated pool to a circulating pool. Reduced *Lias* expression in the Lias heterozygous mice has almost no effect on the initial drop, even though both pro-inflammatory TNF-α and anti-inflammatory IL-10 levels in their plasma are higher than in wild type mice during the early phase. However, the Lias heterozygous mice are severely impaired in the recovery phase. Thus, the WBC counts in these mice at 8 and 16 hours after infection remained low, and inflammatory cell infiltration (Leukocyte extravagation) in the liver and lung were more pronounced than in wild type tissue judged by the tissue MPO

activities and histological assessments. These were accompanied by a significant increase of oxidative stress biomarkers in these tissues at 8 hour after LPS administration. Increased expression of the gene for GRO in the liver suggests that enhanced endothelial activation and recruitment of neutrophils.

The impairment in the process of leukocytes from margination back to circulation in the Lias heterozygous mice suggests that lipoic acid affects proteins involved in the leukocytes trafficking. Reactive oxygen species and other inflammatory mediators including cytokines activate endothelium and up-regulate the density of ICAM-1 and platelet-endothelial cell adhesion moleculae-1 (PECAM-1) and induce exposure of P- and E-selectin on endothelium (Springer, 1990). Thus, we found that gene expression of VCAM-1 was significantly higher in the Lias heterozygous mice livers than the wild type. The expression of genes for ICAM showed a similar trend. In contrast, gene expression of E- or L-selectin was not different. Although the levels of active proteins were not measured, these results suggest than endogenous LA production may affect adhesiveness of endothelial cells for neutrophils/monocytes. This is consistent with the findings by Zhang and Frei who showed that exogenous LA can inhibit TNF-α induced expression of ICAM-1 and VCAM-1 at the mRNA level in cultured human aortic endothelial cells (Zhang and Frei, 2001) and by Chaudhary *et al*. who demonstrated the ability of lipoic acid to inhibit up-regulation of *ICAM-1* and *VCAM-1* in endothelial cells on central nervous system in mice (Chaudhary *et al*., 2006) although the detailed anti-inflammatory mechanisms of lipoic acid remain elusive.

Thrombocytopenia commonly takes place in septic shock due to peripheral abnormalities associated with platelet loss or increased destruction of margination of activated platelets into the peripheral circulation (Stephan *et al*., 1999; Vincent *et al*., 2002).

LPS has been shown to increase platelet aggregation in animal model (Itoh *et al*., 1996; Sheu *et al*., 1999). Noticeably, neutrophil infiltration in lung of LPS treated Lias heterozygous mice is consistent with a report by Strange *et al*. that platelets attenuate oxidant-induced permeability in endothelial cells in glutathione-dependent mechanism (Strange *et al*., 1996). This mechanism may be also account why both platelets and WBC shared the similar recovery curve at 4 hour after LPS injection in wild type mice. Recently, Andonegui *et al*. reported that platelets express functional levels of TLR4 ad that the presence of TLR4 on platelets is essential for the LPS induced thrombocytopenia (Andonegui *et al*., 2005). The authors also demonstrated that LPS induced platelet sequestration is entirely dependent upon neutrophil accumulation into the lung.

Activated leukocytes and macrophages trigger a large amount of ROS production during sepsis. Glucose uptake of these cells increases through membrane translocation of Glut1 and its transcriptional up-regulation. The energy in these mitochondria-poor cells is then provided by the hexose-monophosphate shunt with pyruvate and lactate as end products. Alteration of glucose metabolism in cells, such as hepatocytes, also takes place concurrently with increased expression of genes for glycolytic enzymes and reduction of key enzymes in TCA cycle. Thus, pyruvate dehydrogenase (PDH) enzyme activity reduces through phosphorylation of the subunit E1 to an inactive form by increased PDH kinase activity. LPS challenge also results in transcriptional suppression of the PDH-E1 and E2 genes in various organs (Kim et al., 2006). Since the major source of the cellular ROS is through oxidative phosphorylation in mitochondria, reduction of oxidative metabolism is consistent with the body's attempt to cope with an increased oxidative stress. In the current work, we found that that LPS markedly decreased the Lias gene expression. Since LA is a mandatory cofactor for

PDH and a-KDH, reduction of Lias also ensures the reduction of oxidative metabolism under sepsis. This metabolic change induced by LPS is the same as switch required for cellular adaptation to hypoxia, though whether sepsis induces hypoxic condition or not has been under continual debate, hypoxia-inducible factor 1a has been implicated to activation of genes such as glucose transporters, glycolytic enzymes, PDH kinase and vascular endothelial growth factor. Although further studies are necessary to elucidate the mechanism underlying the down regulation of the Lias gene expression in sepsis, one adverse consequence of this down regulation is the reduction of cellular antioxidant capacity, and our immunostaining of the liver sections provides the evidence for the profound reduction of tissue LA in the Lias heterozygous mice. This further contributes to the exacerbation of tissue damage.

In summary, the present study shows that the Lias heterozygous mice are abnormally sensitive to LPS induced inflammatory tissue damage and suggests that endogenous LA as an antioxidant reduces pathological changes of inflammation in liver and lung possibly through quenching ROS. These results provide additional evidence that oxidative stress is an important factor in septic shock and basis that antioxidant therapy can be an alternative treatment for endotoxin infection.

CHAPTER 3

ASSESSMENT OF ISOPROTERENOL AND PHENYLEPHRINE INDUCED PATHOPHYSIOLOGY IN LIPOIC ACID SYNTHASE DEFICIENT MICE

3.1 INTRODUCTION

Cardiac hypertrophy is associated with other forms of cardiovascular diseases such as hypertension, cardiomyopathy, and heart failure, and chronic pressure and/or volume-overload to the heart followed by prolonged adaptation period often results in cardiac hypertrophy (Grossman *et al*., 1975; Bohm *et al*., 1997; Frey, 2004). Although chronic pressure and volume overload can lead to cardiac hypertrophy, its dynamic and diverse etiology remains elusive. Despite its diverse mechanisms and stimuli, altered metabolism in which metabolic shift from fatty acid oxidation to glucose oxidation in the cardiomyocytes is one distinctive characteristics of this disease (Barger and Kelly, 1999; Leong *et al*., 2002; Bilsen *et al*., 2003).

Several investigations observed enhanced activity of sympathetic nervous system targets many downstream signaling pathways and regulates transcriptional activities lead to the progression of hypertrophy in animal models (Saadane *et al*., 1999, 2000; Iaccarino *et al*., 1999). Adrenergic receptor (adrenoceptor) activation mediates the effects of epinephrine by various agonistic and antagonistic pathways and can be classified into two types, alpha and beta which the former produces excitatory responses of smooth muscle whereas the latter induce inhibitory responses of smooth muscle (Heinsimer *et al*., 1982; Lefkowits *et al*.,

1984). Isoproterenol (ISO) is one of beta-1 adrenocepters known to produce inhibitory effect as beta-1 agonists on smooth muscle, but it produces excitatory effect on the cardiac muscle (Heinsimer *et al*., 1982; Lefkowits *et al*., 1984). Phenylephrine (PE) is one of alpha-1 adrenocepter agonist which induce vasoconstriction and inhibition of cAMP production in cells. Both adrenergic receptor agonists have been used widely as stimuli to induce hypertrophy and other cardiovascular complications (Saadane *et al*., 1999, 2000; Iaccarino *et al.,* 1999; Shannon *et al*., 2006; Shizukuda *et al*., 1998).

Lipoic acid is a crucial component of pyruvate dehydrogenase (PDH) and alpha-ketogluterate dehydrogenase (KDH) enzyme complexes in glucose oxidation pathway and production of ATP. Several investigations with animal models of cardiovascular diseases including hypertrophy and cardiac failure have suggested that the dearth of conserved energy due to aberrant metabolism is associated with abnormal cardiac output resulting in cardiac complications (Huss and Kelly, 2005; Bilsen *et al*., 2003; Ventura-Clapier *et al*., 2003; Leong *et al*., 2003). Reduced enzymatic activities due to reduced lipoic acid synthase gene activity may results in altered metabolism in the heart and reduced cardiac energy output.

In this study, I tested hypothesis that reduced lipoic acid synthase gene activity increases susceptibility to cardiovascular diseases such as hypertrophy and cardiac failure upon cardiac specific adrenergic agonist stimulation. Heterozygous Lias deficient mice and wild type littermates were stimulated with isoproterenol and phenylephrine, cardiac specific adrenergic agonists for two weeks, to increase heart rate and to induce hypertrophy. Our data demonstrate that both genotypes equally developed cardiac hypertrophy with increased HW/BW ratio, and enhanced fibrosis. However, reduced lipoic acid synthase activity appears

to be adaptive and protective against mortality associated with isoproterenol and phenylephrine induced cardiac hypertrophy.

3.2 MATERIALS AND METHODS

3.2.1 Mice and Adrenergic Agonist Administration

Experiments were conducted in the 5-6 months old F1 (C57/Bl6 and 129 SvEv) Lias heterozygous and wild type littermates. Both females and males were used. Isoproterenol bitartrate salt dehydrate and phenylephrine hydrochloride (Sigma-Aldrich, St Louis, MO) were dissolved in PBS and delivered by the ALZET mini-osmotic pump (30mg/kg/bw each, model #2002, DURECT Corporation, Cupertino, CA). Pump was surgically implanted dorsally through a small incision under the skin of anesthetized (1-4% oxygenated isoflourene) mice and continuously infused for 14 consecutive days (0.5µl per hour). Control groups received PBS.

3.2.2 ECHO Cardiogram

ECHO cardiogram (Visualsonics, Vevo Model #660, Toronto, Ontario) was performed on conscious mice at two time points, before and after implantation. Parameters such as PWSP (left ventricular posterior wall thickness septal line), IVS (intra ventricular septum thickness), LVID (left ventricular internal dimension), LV Vol (left ventricular volume) at systolic and diastolic were measured and LV % FS (left ventricular percent fractional shortening) and LV % EF (left ventricular percent ejection fraction) were calculated. All measurements were performed on conscious and strained mice.

3.2.3 Body Weight and Heart Weight Measurement

Body weight was measured before and after pump implantation. For the heart weight, mice were fasted approximately 3 to 4 hours and anaesthetized with an overdose of avertin (2.5%, I.P.). Heart was surgically removed, rinsed in PBS and blotted gently on paper and weighed. The heart to body weight ratio was calculated and determined accordingly.

3.2.4 RNA Isolation and Gene Expression Analysis

Heart tissue was transferred immediately after surgical removal and stored in RNA later solution (Ambion, Austin, TX) overnight. RNA isolation was done by the ABI 6700 RNA automated robot, approximately 30 to 100mg of tissue was weighed and was put into 1ml of lysis buffer (ABI, diluted with 1X PBS). Samples were then homogenized thoroughly for 30 seconds for two times. Cooled down for five minutes and stored overnight at 4°C. Roughly, 10 to 20µl of proteinase K (10ug/µl) was added into lysate, incubated at room temperature for an hour and then cooled down at ice for five minutes. Approximately, 200µl out of 1ml of lysate was applied to ABI 6700 RNA extraction robot. Remaining steps were followed by the manufactures' protocol. Isolated RNA was analyzed for marker genes that are for hypertrophy. The relative levels and patterns of genes-*MCAD, β-MHC, ANP, Collagen I, Collagen III* and *Lias* were determined by real-time quantitative reverse transcription PCR (RT-PCR) with b-actin as the reference gene in each reaction (Kim *et al*., 2002) and sequence of their primers and probe are summarized in the Table 2.1.

3.2.5 Blood Sample Collection and Metabolite Measurements

Mice were fasted for approximately 4-5 hours before collection. Retro-orbital bleeding was performed with heparinized capillaries to obtain blood from each animal.

Collection tubes were anti-coagulated with 3µl of 0.5M of EDTA and whole blood was then centrifuged at 1,600g for 10 minutes at 4°C to separate the plasma. Plasma levels of glucose (WAKO, Richmond, VA), triglyceride (Stanbio, Boernl, TX), cholesterol (WAKO, Richmond, VA) and free fatty acid (WAKO, Richmond, VA) were determined by enzymatic reaction of 2µl of plasma in 200µl of commercially available solution followed by spectrophotometer quantitation. A portion of frozen heart tissue was homogenized in 1ml of ice-cold 50mM potassium phosphate buffer (pH 7.4) and then centrifuged at 4°C of 10,000g for 30 minutes. The level of oxidative stress was assessed by measuring the level of lipid peroxidation, TBARS (Thiobarbituric Reactive Substances) in plasma and heart tissue homogenates (Lapenna *et al*., 2001) and the level of lactate in heart tissue was measured as described (Gutmann *et al*., 1974). Protein concentrations of tissue homogenates were determined based on the Bradford method (Bio-Rad Laboratories, Hercules, CA) and known concentrations of BSA (bovine serum albumin) was used as a standard.

3.2.6 Histological Analysis

Heart was surgically excised, briefly rinsed in PBS and blotted on the paper. Heart was then cut in half horizontally and mid-top portion of the heart was immediately kept in 4% buffered PFA (pH 7.4) solution, and then fixed overnight. Samples were trimmed next day for proper size and orientation and sent to the Histological Core Facility at University of North Carolina at Chapel Hill for further process. Three to five micron sectioned heart slides were stained with H&E (hematoxylin and eosin) and Masson's trichrome staining.

3.2.7 Statistical Analysis

Statistical analyses were carried out with JMP software (SAS, Cary, NC). Values reported here is mean ± SEM. Effect of genotype and drug treatment was analyzed by two-factorial ANOVA followed by Tukey-Kramer test. Student's *t* test was used for comparison between heterozygous and wild type mice group. $P<0.05$ was considered as a statistical significance.

3.3 Results

3.3.1 Survival of the Lipoic Acid Synthase Deficient Mice after ISO+PE Stimuli

During the 14 days infusion period, survival rate of mice in four groups (wild type group, treated and sham; Lias heterozygous group, treated and sham) were recorded. As seen in Figure, 3.1., the survival curves (percentile scale) of four groups were relatively remained similar by the day 7 (Lias+/- treated=92%, control=91%; Lias+/+ treated=80%, control=88%). Surprisingly, the survival rate of the wild type group with treatment dramatically decreased during day 7 to 14 whereas Lias heterozygous mice with treatment remained relatively higher (Lias+/- treated=70%, control=91%; Lias+/+ treated=20%, control=88%) which indicates that Lias heterozygous deficiency appears to be protective against isoproterenol and phenylephrine adrenocepter agonists stimulus compared to the wild type mice. The other two control groups of the both genotypes (Lias+/- control=91%; Lias+/+ control=88%) showed minimal loss during the experiment, and no significant reduction of viability was observed until the end of experiment.

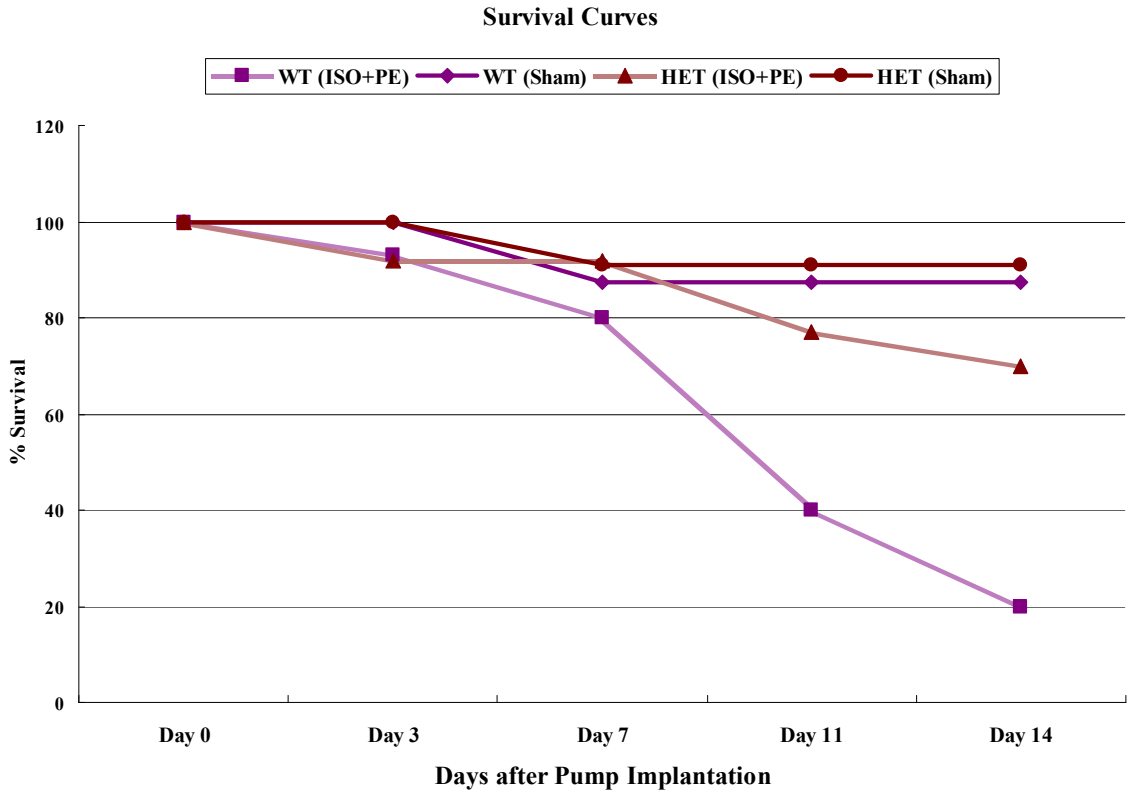


Figure 3.1. Survival curves of four experimental groups after isoproterenol and phenylephrine administration. Percentile survival rate was determined before and during the experiment. The number of alive mice was divided by the total number of mice in each group (death of post surgery was also counted in calculation). Number of mice, n=15 (wild type treated), n=8 (wild type sham), n=13 (heterozygous treated), n=11 (heterozygous sham).

3.3.2 Body Weight, Heart Weight and HW/BW Ratio after ISO+PE Stimuli

I measured the body weight of all mice before and after the treatment and heart weight after treatment. As seen in Table 3.1., the body weight was decreased (changes represented as delta) after the treatment in both genotypes (Lias +/- treated =-2.4±0.98g, control=0.38±0.7g; Lias +/+ treated=-2.3±1.2g, control=0.65±0.6g; genotypic effect, no significance; treatment effect, $P<0.01$) and their HW/BW ratio markedly increased after the treatment (Lias +/- treated=0.80±0.04, control=0.52±0.02; Lias +/+ treated=0.91±0.06, control=0.52±0.01; genotypic effect, no significance; treatment effect, $P<0.0001$). Thus, the 14 days of stimulation of alpha-1 and beta-1 adrenocepter with isoproterenol and phenylephrine resulted in decreased body weight and increased HW/BW ratio of all treated mice. However, a genotypic effect of Lias deficiency after isoproterenol and phenylephrine treatment was not observed.

Table 3.1.

Heart weight, body weight and their ratio

Parameters	WT(Sham)	WT(Treat)	Lias+/-(Sham)	Lias+/-(Treat)
BW (g)-before	33.1±2.1	31.8±1.5	28.7±2.0	29.3±1.1
BW (g)-after	33.5±2.5	29.9±1.3	29.6±2.4	26.9±2.6
ΔBW (g)	0.65±0.6†	-2.3±1.2†	0.38±0.7††	-2.4±0.98††
HW (g)-after	0.17±0.01‡	0.27±0.02‡	0.15±0.02‡‡	0.22±0.02‡‡
HW/BW ratio	0.52±0.01*	0.91±0.06*	0.52±0.02**	0.80±0.04**

†, P<0.01, Treatment effect on wild type; ††, P<0.01, Treatment effect on Lias heterozygous; ‡, P<0.01, Treatment effect on wild type; ‡‡, P<0.05, Treatment effect on Lias heterozygous; *, P<0.0001, Treatment effect on wild type; **, P<0.0001, Treatment effect on Lias heterozygous.

Table 3.1. Body weight, heart weight and their ratio before and after isoproterenol and phenylephrin administration. Body weight of all groups was measured before and after ISO+PE administration. The body weight (changes represented as delta) decreased on both genotypes (no genotypic effect). The heart weight increased after treatment on both genotypes (no genotypic effect), and relative heart and body weight ratio of both groups significantly increased after treatment (no genotypic effect); Wild type (sham, n=7; treat, n=12), Lias heterozygous (sham, n=10; treat, n=13).

3.3.3 ECHO Cardiogram

I also examined the functionality of the heart by applying ECHO cardiogram before and after the treatment of all groups. As seen in Figure, 3.2 and 3.3, I observed increased thickness of the IVS (intraventricular septum) at diastolic in the treated Lias heterozygous group compared to Lias heterozygous control group (Lias +/- treated=0.6±0.13mm; Lias +/- control=-0.04±0.24mm, treatment effect, $P<0.05$) whereas wild type did not change upon treatment (Lias +/+ treated=0.45±0.22mm; Lias +/+ control=0.17±0.18mm, treatment effect, not significant). Also, IVS at systolic in the both groups markedly increased on both genotypes (Lias +/- treated=0.27±0.14mm, Lias +/- control=-0.19±0.2mm; Lias +/+ treated=0.44±0.12mm, Lias +/+ control=-0.01±0.16mm; treatment effect, $P<0.01$; genotype effect, not significant) upon the stimulus, but did not detect genotypic difference. I did not observe genotypic nor treatment effect on other parameters such as PWSP (left ventricular posterior wall thickness at diastolic and systolic), LVID (left ventricular internal dimension at diastolic and systolic), LV Vol (left ventricular volume at diastolic and systolic), LV % FS (left ventricular percent fractional shortening) and LV % EF (left ventricular percent ejection fraction) but observed trends of decreased LVID at diastolic and systolic and LV Vol at diastolic and systolic after stimulation for the both genotypes. The heart rate of four mice groups at before, after the ISO+PE stimulation and the difference (delta) are also assessed as seen in Table 3.2. The heart rate of all mice groups before and after agonist stimulation does not differ from each other. Moreover, the difference (represented as delta) of all mice groups was not statistically significant.

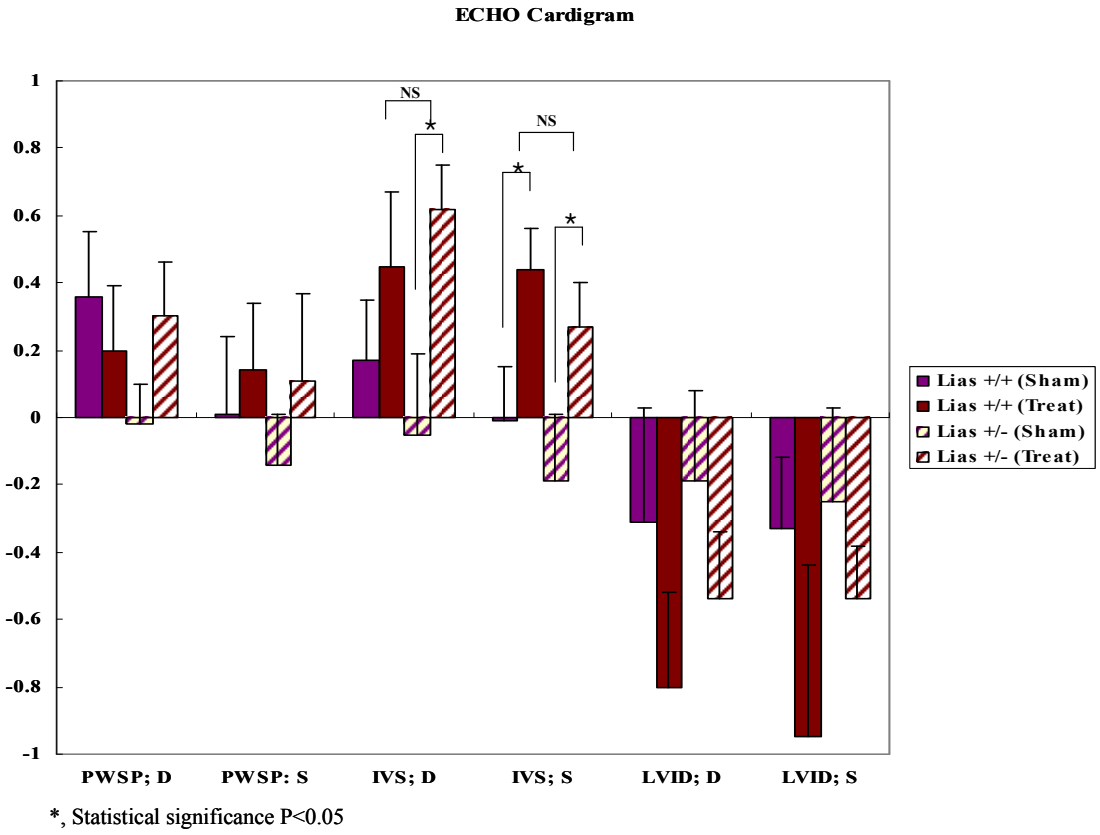


Figure 3.2. ECHO Cardiogram before and after isoproterenol and phenylephrine administration. ECHO cardiogram measurements before and after ISO and PE administration. Changes represented as delta values. PWSP (left ventricular posterior wall thickness septal line at diastolic and systolic), IVS (intra ventricular septum thickness at diastolic and systolic), LVID (left ventricular internal dimension at diastolic and systolic) were measured. After 14 days infusion, thickness of IVS at systolic and diastolic has increased in Lias heterozygous and wild type due to treatment, however, genotypic effect was not observed. Wild type (Sham, n=5; treat, n=7), Lias heterozygous (Sham, n=4; Treat, n=9), a unit is represented as millimeter.

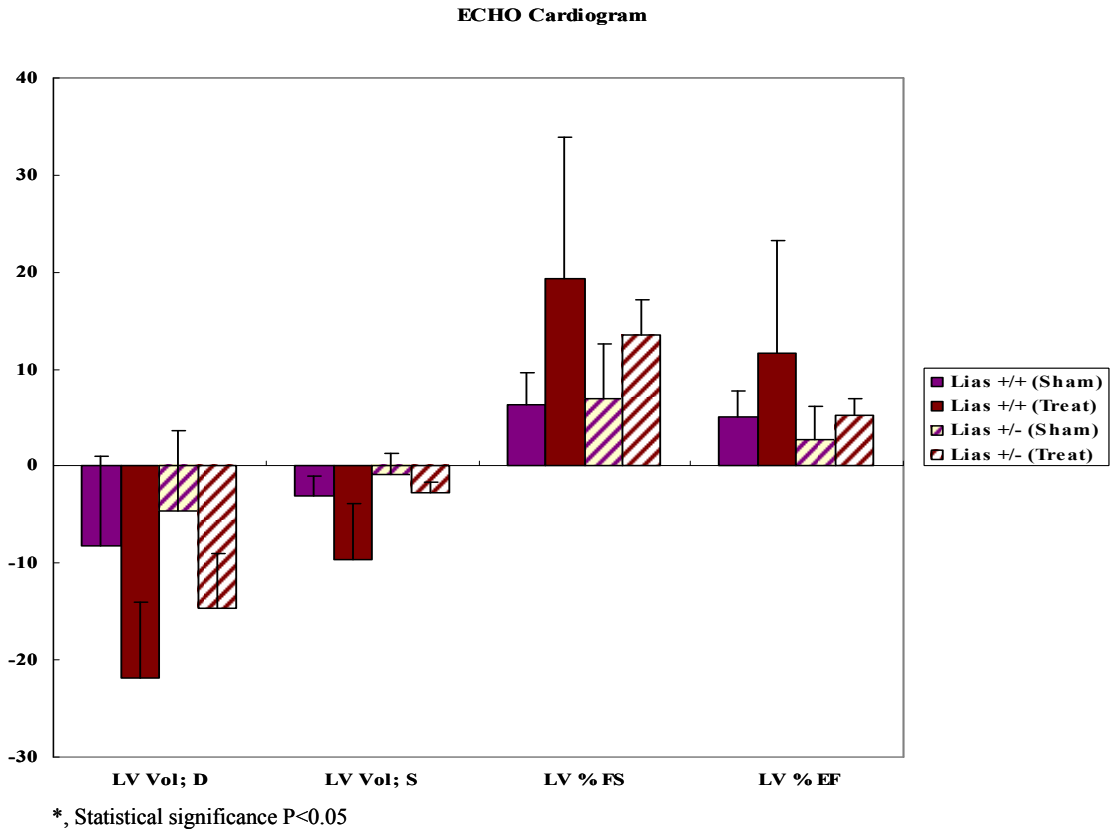


Figure 3.3. ECHO Cardiogram before and after isoproterenol and phenylephrine administration. ECHO cardiogram measurements before and after ISO and PE administration. Changes represented as delta values. LV Vol (left ventricular volume at diastolic and systolic), LV % FS (left ventricular % fractional shortening), LV % EF (left ventricular % ejection fraction) were measured. After 14 days infusion, both genotypes show trends of decreased left ventricular volume with increased % fractional shortening and ejection fraction, however, did not reach statistical significance. Wild type (Sham, n=5; treat, n=7), Lias heterozygous (Sham, n=4; Treat, n=9), a unit is represented as ml in volume.

Table 3.2.
Heart rate measurement before and after stimulation

Parameters	WT(Sham)	WT(Treat)	Lias+/-(Sham)	Lias+/-(Treat)
HR-before	602±31	570±33	647±18	609±30
HR-after	723±16	658±58	666±78	730±14
ΔHR	122±35	120±72	-13.8±76	128±44

Table 3.2. ECHO Cardiogram – Heart rate changes before and after isoproterenol and phenylephrine administration. Heart rate was measured before and after ISO and PE administration. Unexpectedly, heart rate of all groups was increased; however, the changes (represented as delta) were not different from sham and treated groups except heterozygous sham group.
Wild type (Sham, n=6; Treat, n=15), Lias heterozygous (Sham, n=9; Treat, n=13) for the heart rate measurement before treatment; wild type (Sham, n=6; Treat, n=6), Lias heterozygous (Sham, n=4; Treat, n=9) for the heart rate measurement after treatment
Unit is represented as beat per minute (BPM).

3.3.4 Plasma Metabolites, TBARS and Lactate Levels after ISO+PE Stimuli

Next, I determined the plasma levels of the glucose, triglyceride and cholesterol at the baseline and after stimulation to monitor their metabolic changes in the whole body over the experimental period. Table 3.3. represents overall changes (delta) in plasma glucose, triglyceride, cholesterol and FFA (free fatty acid) during the experiment, and I observed decreased levels of blood glucose in the wild type group compared to Lias heterozygous mice after treatment (Lias +/- treated=-13.0±15.0mg/dl, control=14.5±13.6mg/dl, no treatment effect; Lias +/+ treated=-33.8±3.4mg/dl, control=61.9±16.2mg/dl, treatment effect, $P<0.01$). The plasma triglyceride levels did not differ from genotypes after stimulation (Lias +/- treated=-18.3±10.8mg/dl, control=-22.5±7.5mg/dl, Lias +/+ treated=-3.8±1.5mg/dl, control=-20.8±9.6mg/dl, no treatment effect, no genotype effect), and the cholesterol levels did not differ from genotypes before and after the treatment (Lias +/- treated=8.6±6.2mg/dl, control=17.8±3.3mg/dl, Lias +/+ treated=13.0±11.0mg/dl, control=27.1±4.2mg/dl, no treatment effect, no genotype effect). I also measured FFA (free fatty acid) after the adrenergic agonist stimulation whether the chronic infusion over two weeks influence FFA levels in the plasma, but did not detect any genotypic nor treatment effect on all experimental groups (Lias +/- treated=1.22±0.05mEQ/L, control=1.28±0.06mEQ/L, Lias +/+ treated=1.17±0.02mEQ/L, control=1.25±0.06mEQ/L, no treatment effect, no genotype effect).

Isoproterenol and phenylephrine are adrenocepter agonists that stimulate and excite the cardiac functions. Chronic stimulation act as chemical stress, and often results in cardiac toxicity (Ishizawa *et al*., 2006; Rajadurai *et al*., 2006; Diaz-Munoz *et al*., 2006), so we assessed the plasma and tissue levels of oxidative stress by measuring the lipid peroxidation

(TBARS) in all four experimental groups. As seen in Table, 3.3., plasma levels of TBARS were not detected due to low serum levels of oxidative stress in all experimental groups. However, I observed significantly decreased tissue levels of TBARS in the Lias heterozygous mice group after stimulation (Lias +/- treated=0.06±0.02nmol/mg protein, control=0.09±0.01nmol/mg protein, treatment effect, $P<0.05$; Lias +/+ treated=0.05±0.01nm/mg protein, control=0.06±0.01nmol/mg protein, no treatment effect). As expected, levels of TBARS in the Lias heterozygous control group increased compared to wild type control group (Lias +/- control=0.09±0.01nmol/mg protein, Lias +/+ control=0.06±0.01nmol/mg protein, genotype effect, $P<0.05$, no treatment effect).

Lias gene is essential for energy metabolism in the mitochondria. A previous study showed Lias heterozygous mice have altered metabolism with increased levels of lactate in liver tissue may be due to the enhanced glycolytic activity (Yi *et al*., 2005). Several animal studies have indicated that altered metabolism from the fatty acid oxidation to carbohydrate and glucose oxidation is associated with the hypertrophy although whether this metabolic switch is adaptive or maladaptive is under debates (Huss and Kelly, 2005; Ventura-Clapier *et al*., 2003; Bilsen *et al*., 2003). In order to determine whether the enhanced glycolytic activity of the Lias heterozygous mice shows altered metabolism in the heart upon the ISO and PE stimulation, I measured the lactate levels in the heart tissue. As seen in Table 3.3., both genotypes show significantly decreased level of lactate in the heart tissue after the stimulation, however, genotypic difference was not observed (Lias +/- treated=0.43±0.12mmol/mg protein, control=0.68±0.02mmol/mg protein, treatment effect, $P<0.01$; Lias +/+ treated=0.31±0.07mmol/mg protein, control=0.61±0.04mmol/mg protein, treatment effect, $P<0.05$).

Table 3.3.

Measurements of biomarkers after treatment in plasma and heart tissue

Parameters	WT(Sham)	WT(Treat)	Lias+/-(Sham)	Lias+/-(Treat)
ΔGlucose(P)	61.9±19.2**	-38.8±3.4**	14.5±13.6	-13.0±15.0
ΔTG(P)	-20.8±9.6	-3.8±1.5	-22.5±7.5	-18.3±10.8
ΔCholesterol(P)	27.1±4.2	13.0±11.0	17.8±3.3	8.6±6.2
FFA(P)	1.25±0.06	1.17±0.02	1.28±0.06	1.22±0.05
TBARS(P)	ND	ND	ND	ND
TBARS(H)	0.06±0.01‡	0.05±0.01	0.09±0.01†‡	0.06±0.01†
Lactate(H)	0.61±0.06*	0.31±0.08*	0.68±0.07†	0.43±0.07†

Table 3.3. Measurement of metabolites in plasma and heart tissue before and after isoproterenol and phenylephrine administration. Plasma levels of glucose, triglyceride, cholesterol, free fatty acid and TBARS and tissue levels of lactate and TBARS were measured before and after ISO and PE administration. Significant reduction of glucose level in treated wild type was detected compared to Lias heterozygous mice. Level of TBARS and lactate in heart tissue of both genotypes lowered after ISO and PE administration. Glucose, triglyceride, cholesterol and FFA levels: wild type (Sham, n=8; treat, n=3), Lias heterozygous (Sham, n=10; Treat, n=9), unit in glucose, triglyceride and cholesterol is represented as mg/dl, unit in free fatty acid is represented as mEq/L. TBARS and lactate levels: wild type (Sham, n=6; treat, n=3), Lias heterozygous (Sham, n=4; Treat, n=4), unit in TBARS is represented as nmol/mg protein in tissue, and nmol/ml in plasma, unit in lactate is represented as mmol/mg protein; (P), plasma levels of metabolites; (H), tissue (heart) levels of metabolites.

**, $P<0.01$, treatment effect on wild type (Sham vs Treat); *, $P<0.05$, treatment effect on wild type (Sham vs Treat); †, $P<0.05$, treatment effect on Lias heterozygous (Sham vs Treat); ‡, $P<0.05$ genotype effect (wild type vs Lias heterozygous); ND, not determined.

3.3.5 Enhanced Expression of Hypertrophic Marker Genes and Increased Fibrosis after ISO+PE Stimuli

Many animal studies have shown the effects of the ISO and PE administration on promoting hypertrophy and congestive heart failure by stimulating various downstream sympathetic pathways and target genes (Saadane *et al*., 1999, 2000). Expression of several genes including *MCAD*, *β-MHC* and *ANP* (*ANF*) are known to be altered during the process and used as indicators of the hypertrophy (Barger and Kelly, 1999). The relative mRNA expression of *MCAD* was decreased after agonist stimulation on both genotypes (Lias +/- treated=55.0±10.0, control=100±10.3, Lias +/+ treated=53.0±9.5, control=100±12.4, treatment effect, $P<0.0001$, no genotypic effect) (Fig 3.4.). The expression level of *β-MHC* in the treated heterozygous group increased compared to the treated wild type mice group (Lias +/- treated=233±41, control=71.4±43, Lias +/+ treated=76.2±38, control=100±53, treatment/genotype effect was observed, $P=0.05$). *ANP* expression level of both genotypes tended to increase upon the isoproterenol and phenylephrine stimulation (Lias +/- treated=119±19.4, control=95.2±20.4, Lias +/+ treated=120.2±16.7, control=100±24.3, no significant treatment/genotype effect was observed), but both genotypes did not reach statistical significance after infusion. Relative mRNA levels of *Collagen I* (Lias +/- treated=421±155, control=116±163, Lias +/+ treated=709±139, control=100±194, no genotypic effect; treatment effect, $P<0.01$) and *Collagen III* (Lias +/-, treated=212±53.8, control=105±56.4, Lias +/+, treated=344±51.5, control=100±67.3, no genotypic effect; treatment effect, $P<0.01$), markers for fibrosis in the heart increased significantly upon the treatment. These data from both groups of mice implicates that ISO and PE stimulus up-regulated *Collagen I* and *Collagen III* mRNA expression which generally results in enhanced

fibrosis formation. The levels of *Lias* expression was not changed after ISO and PE induced cardiac stress on both genotypes (Lias +/-, treated=51.5±18, control=49.4±9.9, Lias +/+, treated=86.3±8.9, control=100±11.8, genotypic effect as expected; no treatment effect).

Next, I assessed fibrosis formation in the heart by evaluating Masson's trichrome stained heart sections (arbitrary scores were measured with degree of severity scales from 0 to 3). As seen in Figure 3.5. and 3.6., both Lias heterozygous and wild type mice groups showed increased fibrosis in their heart after isoproterenol and phenylephrine stimulation (Lias +/- treated=2.12±0.23, control=0.65±0.48, Lias +/+ treated=2.08±0.27, control=0.83±0.11, no significant genotype effect was observed; treatment effect, $P<0.001$). These data indicates that both genotypes responded upon isoproterenol and phenylephrine cardiac stimulation regardless of their sex, and 14 days of consecutive infusion induced cardiac hypertrophy and cardiac fibrosis in treated mice..

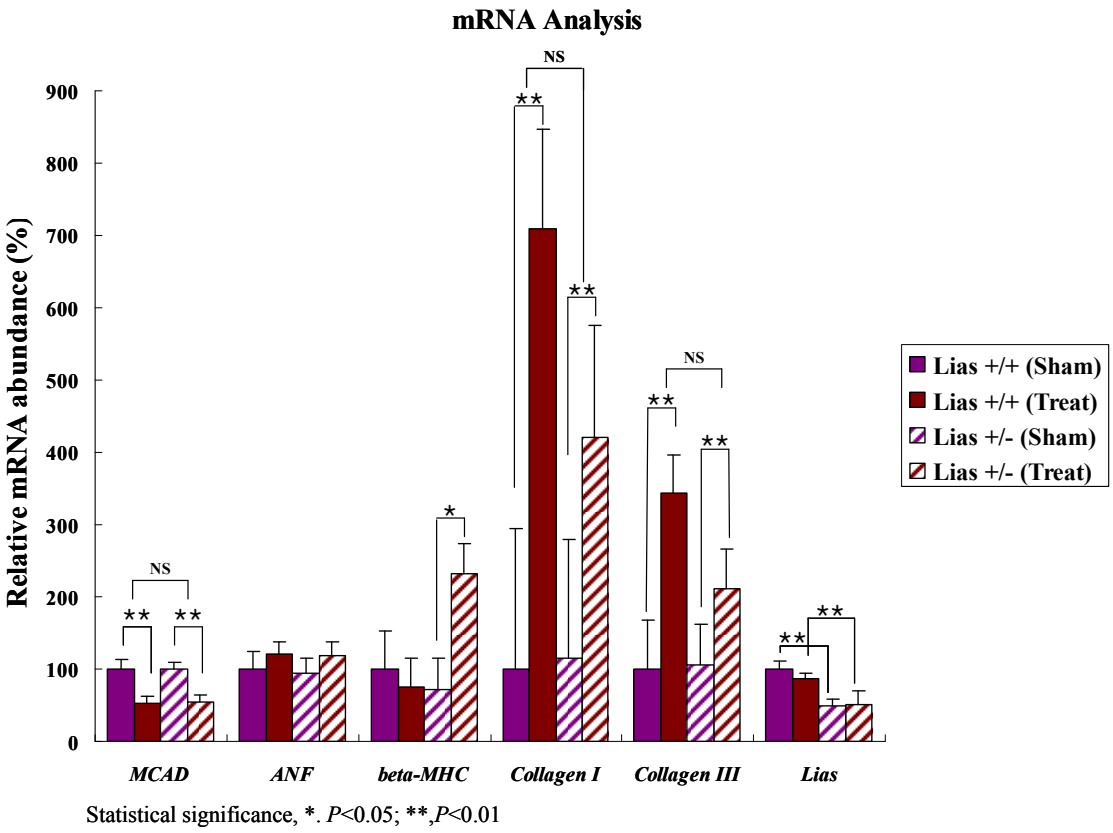


Figure 3.4. Relative gene expression in heart after isoproterenol and phenylephrine administration. After 14 days of consecutive infusion of ISO and PE, both treated groups showed significant decrease in *MCAD*, increase in *Collagen I* and *III* expression (no genotype effect was observed; treatment effect, $P<0.01$). The expression levels of *ANF* tended to increase after treatment; however, it did not reach statistical significance. Treated Lias heterozygous group showed increased level of *β-MHC* expression in the heart (genotype/treatment effect was observed, $P=0.03$). As expected, *Lias* gene expression is reduced in Lias heterozygous mice compared to wild type mice, but the relative levels of both genotypes did not change after treatment.

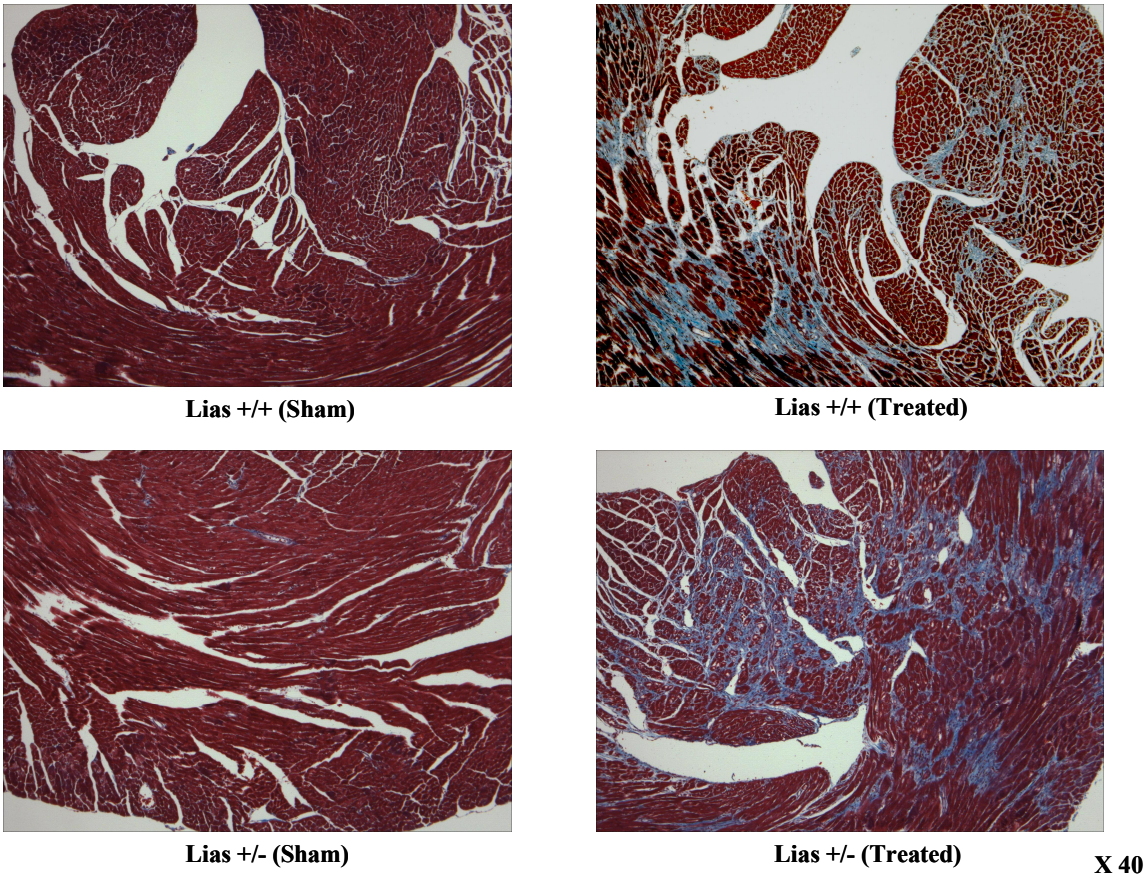
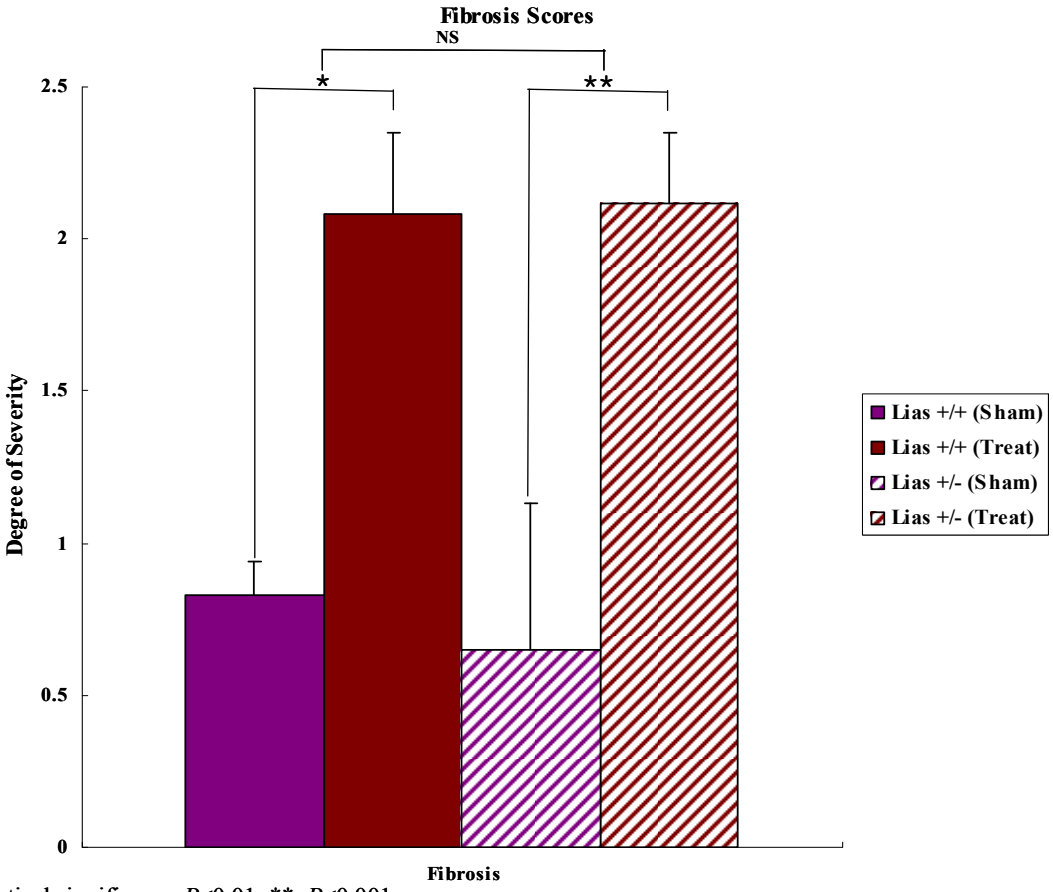


Figure 3.5. Histological analysis of heart after ISO+PE administration. The heart sections stained with Masson's trichrome, an indicator of fibrosis. After 14 days of consecutive ISO+PE administration, both treated groups developed mild to severe fibrosis in their hearts, whereas sham groups developed no fibrosis. However, no genotypic effect was observed.



*, Statistical significance $P<0.01$; **, $P<0.001$

Figure 3.6. Arbitrary scores of heart fibrosis after ISO+PE administration. Arbitrary scores of heart fibrosis from the fig. 3.5. Each slide was individually analyzed and arbitrary score of severity was given (severity scales are from 0, none, to 3, severe). Then, average was calculated for all groups. After 14 days of consecutive isoproterenol and phenylephrine administration, both treated groups developed mild to severe fibrosis in their hearts, whereas sham groups did not develop fibrosis. However, no genotypic difference was observed.

3.4 DISCUSSION

In this study, I have observed reduced viability of wild type mice with isoproterenol and phenylephrine infusion over the 14 days experiment. Surprisingly, I observed a protective effect of Lias heterozygosity compared to wild type littermates upon stimulation. Unlike other isoproterenol and pheylephrine related animal experiments, I observed significantly enhanced mortality in the wild type (20% survival rate) and a protective effect of Lias heterozygous deficiency (70% survival rate) against adrenergic stress, although the concentration of these two drugs was smaller than the known LD_{50} (Green *et al*., 1980).

Isoproterenol and phenylephrine are adrenergic agonists. Isoproterenol is beta-adrenergic receptor agonist which has known to produce systemic vasodialation, bronchodilation, and hypotension effects (Heinsimer *et al*., 1982; Lefkowitz *et al*., 1984). Pheylephrine is alpha-adrenergic receptor agonist which has known to induce vasoconstriction, bronchodilation, and hypertension effects (Heinsimer *et al*., 1982; Lefkowitz *et al*., 1984). Several investigations have observed synergistic effects of increased cardiac outputs when both drugs are used together (Shannon and Chaudhry, 2006; Saadane *et al.*, 1999; Priori *et al.*, 1993), and continuous infusion by a mini-osmotic pump resulted in dramatic cardiac phenotypes such as hypertrophy and congestive heart failure in animals (Yutzey and Robbins, 2007; Shannon and Chaudhry, 2006; Leenen, 1999; Singal, 1982; Mann and Bristow, 2005).

Observations from my osmotic mini pump experiments indicate that both wild type and Lias heterozygous mice responded to and developed hypertrophy upon isoproterenol and pheylephrine stimulation. Both treated groups lost total body weight and their heart weight dramatically increased compared to their respective control groups. Although I did not detect

any significant genotypic effect after stimulus in several parameters of the ECHO cardiogram, I observed trends of hypertrophy in all treated groups compared to control groups (increased ventricular wall thickness, decreased left ventricular internal dimension and left ventricular volume). However, overall changes (delta) of parameters such as heart rate, left ventricular percent ejection fraction, and percent fraction shortening were relatively the same regardless of the genotypes and treatment. This discordant result of the ECHO cardiogram may be due to difficulties encountered during the measurements of conscious, strained mice and a limited number of surviving mice which prevent us from reaching statistical significance.

The data from the mRNA analysis of *MCAD* also revealed that both genotypes responded and developed cardiac hypertrophy. *MCAD* levels of treated groups were significantly reduced, indicating that their fatty acid oxidation metabolism was relatively equally interrupted upon cardiac stimulation, although I did not detect significantly increased expression levels of *ANP* (*ANF*) after treatment. Interestingly, I detected significant up-regulation of the *β-MHC* mRNA level in Lias heterozygous treated mice (both treatment and genotype effect, $P<0.05$). Cardimyocytes contain two isoforms of myosin heavy chain, alpha- and beta. *β-MHC* is an isoform which is predominantly present in adult hearts whereas *α-MHC* is present only in developing fetal hearts (Krenz *et al*., 2003, 2004). Transcriptional activities of both isoforms are often considered as sensitive markers for hypertrophy and heart failure (Barger and Kelly, 1999). A previous study from Krenz *et al*. reported that *β-MHC* transgenic mice were perfectly normal with significant reduction in contractile functions in their heart. However, a shift from *α-MHC* to *β-MHC* under the chronic isoproterenol stimulation was disadvantageous although the higher expression of *β-MHC* is

theoretically beneficial and adaptive in a way of preserving energy costs in the heart (Sugiura *et al*., 1998; Holubarsch *et al*., 1985). Since lipoic acid is an essential cofactor for enzyme complexes responsible for oxidative metabolism and ATP synthesis in the mitochondria, I hypothesized that 50% reduction of its gene activity could be detrimental to the initiation and progression of cardiac disease upon chronic adrenergic agonist stimulation. Instead, I observed a protective effect of Lias heterozygous deficiency in this experiment. Perhaps, the up-regulation of the *β-MHC* mRNA levels in the Lias heterozygous mice upon adrenergic stimulation implies that altered metabolism and/or reduced ATP synthesis due to the Lias heterozygous deficiency could be adaptive and or advantageous regarding cardiac energy production and consumption. In other words, a reduction of energy metabolism due to Lias deficiency accompanied with enhanced *β-MHC* expression levels due to adrenergic agonists stimulation by isoproterenol and phenylephrine in the heart can be adaptive and advantageous in this experiment. Currently, the questions remain elusive as to whether the altered metabolism is adaptive or maladaptive in the progression of cardiac dysfunction, and further assessment in regards to *Lias* deficiency upon variety of cardiac stimulation is required.

Our analyses of metabolites in the plasma and heart tissue also indicate that treated wild type mice had significantly reduced plasma glucose levels when compared to treated heterozygous mice, although the number of analyzed wild type mice was small. Interestingly, I observed that the death of treated wild type mice occurred during the fasting state in the middle and near the end of experiments, whereas treated heterozygous mice group were nearly healthy under the same environmental conditions. Several studies reported that acute stimulation of alpha- and beta- adrenergic receptors by administering isoproterenol and

phenylephrine induced hyperglycemic effect resulted from glycogenolysis from the liver (Shannon and Chaudhry, 2006; Vardanega-Peicher *et al*., 2000). However, pathophysiological influence and effect of prolonged stimulation by isoproterenol and pheylephrine on the metabolism remains unknown to date because glycogenolysis upon the adrenergic stimulation varies with several factors such as dosage, animal models, fasting period and state, ages and sex (Vardanega-Peicher *et al*., 2000; Garcia-Sainz *et al*., 1992; El-Refai *et al*., 1982; Bazotte *et al*., 1989; Studer and Borle, 1982; Katz *et al*., 1987). Although I did not investigate the levels of glycogenolysis in the liver tissue of all experimental groups, it is worthwhile to assess whether there is difference in regulating metabolism and/or other hormonal mechanisms (such as insulin and glucagons secretion) upon chronic stimulation of adrenergic receptors.

I also measured the levels of lactate in the heart tissue to test the hypothesis of whether the altered metabolism due to the Lias heterozygous deficiency is adaptive or maladaptive upon the cardiac stress since previous study showed increased levels of lactate in the Lias heterozygous mice because of the enhanced glycolytic activity (Yi *et al*., 2005). After stimulation, lactate levels in the heart tissue of both treated groups significantly decreased implicating that a shift from fatty oxidation to glycolytic pathways occurred, although a genotypic difference was not observed. Several metabolic enzymes in cardiomyocytes are upregulated and down-regulated upon prolonged cardiac stress. For example, pyruvate dehydorgenase complex is a multi enzyme responsible for oxidative metabolism and it is regulated by several kinase and phosphatase isoforms. A recent study from Leong *et al*. investigated activities of PDC and its kinase/phosphates isoforms in hypertrophied and non-hypertrophied hearts, and whether the altered metabolism accounts

for the progression of heart diseases. They speculated that other possible molecular mechanisms of cardiac hypertrophy due to abnormal metabolism (pyruvate transport, NADH shuttles, lactate dehydrogenase and amino acid metabolism) leads to cardiac hypertrophy.

I was unable to detect the plasma levels of lipid peroxidation (TBARS) before and after isoproterenol and phenylephrine stimulation, probably due to the insensitivity of the assay. However, I detected markedly lower levels of lipid peroxidation (TBARS) upon stimulation in the heart tissue after treatment. In the heart tissue, the level of TBARS of the Lias heterozygous control group was higher than the wild type control group as expected. Interestingly, the TBARS levels of both groups reduced after adrenergic stimulation and our data differ from current concepts and experimental results from other investigations (Ishizawa *et al*., 2006; Diaz-Munoz *et al*., 2006; Rajadurai *et al*., 2006). Several studies implicated that excessive alpha- and beta- adrenergic administration or stimulation produce cardiac toxicity and results in reactive oxygen production which will lead to aberrant cardiac energetic (Ishizawa *et al*., 2006; Diaz-Munoz *et al*., 2006; Rajadurai *et al*., 2006). However, it is also proposed that there are powerful counter regulatory and protective mechanisms of cardiomyocytes to reduce toxic effects of agonist stimulation (Shannon and Chaudhry, 2006). Therefore, it is worthwhile to investigate whether the acute or chronic toxicity triggered protective mechanisms of cardiomyocytes upon cardiac stimulation in this study.

In this experiment, I observed the adaptive and advantageous effect of Lias heterozygous deficiency against cardiac specific adrenocepter stimulation. During the fasting state, mortality appeared to be enhanced in the treated wild type mice followed by hypoglycemia, whereas treated Lias heterozygous mice appeared to be normal and healthy. Consequently, adrenergic agonist stimulation induced cardiac hypertrophy on both genotypes

with increased HW/BW ratio, decreased activity of *MCAD*, and increased activity of *Collagen I* and *III* genes accompanied with enhanced fibrosis formation in their hearts.

The question regarding to the death of wild type during this experiment remains unanswered at this point. Perhaps, increased mortality of the wild type groups and protective effect of Lias heterozygous deficiency upon isoproterenol and phenylephrine administration may be an independent phenotype despite overall cardiac changes in this study. For further assessment of effects of the Lias deficiency upon cardiac specific stimulation, several approaches such as a generation of animal models of cardiac specific Lias deficiency, pressure overload TAC aortic banding experiment, and in vitro assays of Lias deficiency with isoproterenol and phenylephrine stimulation experiments can be followed, and these methods will help us to elucidate possible molecular mechanisms and genetic effects of Lias deficiency on the initiation and progression of cardiac abnormalities.

CHAPTER 4

SUMMARY

In this study, I examined the effects of the lipoic acid synthase heterozygous deficiency in regards to initiation and progression of complex human diseases upon exogenous oxidative stress such as lipopolysaccharide, isoproterenol and phenylephrine.

I observed increased sensitivity of the Lias heterozygous deficiency upon LPS induced inflammatory response. In contrast, I observed protective and advantageous effect of Lias heterozygous deficiency upon isoproterenol and phenylephrine although both heterozygous and wild type littermates developed hypertrophy after stimulation. The protective effect of heterozygous deficiency upon isoproterenol and phenylephrine stimulation may not be associated with cardiac specific phenotype, and further investigations should be followed.

Lias is an essential gene responsible for important steps in oxidative metabolism in mitochondria and it is also a candidate gene for metabolic and cardiovascular diseases. For better understanding of the initiation and progression of complex human diseases accompanied by variety of exogenous/endogenous insults, it is worthwhile to further explore the genetic and pathological impacts of Lias deficiency in mouse model.

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