GENETIC REGULATION OF INTRINSIC ENDOTHELIAL FUNCTION AND ENDOTHELIAL RESPONSES TO EXERCISE TRAINING

A Dissertation

by

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Submitted to the Office of Graduate and Professional Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

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December 2015

Major Subject: Kinesiology

ABSTRACT

Endothelial dysfunction is a fundamental component of cardiovascular disease. Exercise training is known to prevent/improve endothelial dysfunction. However, the genetic basis for endothelial function is yet to be fully elucidated and the genetic contribution to endothelial responses to exercise training is largely unknown. The purposes of this research were 1) to identify quantitative trait loci (QTL)/candidate genes residing in the QTL responsible for intrinsic endothelial function and 2) to determine the interaction between genetic background and training intensity on the endothelial adaptations to exercise training. In the first study, vasoreactivity was assessed in aortic rings of male mice from 27 inbred strains. Strain-dependent differences were found for vasoreactivity including responses to ACh. Genome-wide association study for responses to ACh revealed four significant and several suggestive QTL, most of which are regions of shared synteny for cardiovascular traits in rats and/or humans. In the second study, a strain survey for the effect of traditional exercise training on vasoreactivity was performed in aortic rings of male mice from 20 inbred strains. Traditional exercise training had subtle effects on vasoreactivity including responses to ACh. Based on the strain survey, four inbred mouse strains (129S1, B6, SJL, and NON) were chosen to examine endothelial responses to two different training intensities [high (HIT) *vs.* moderate intensity (MOD)]. There was a significant interaction between mouse strain and training intensity on responses to ACh after exercise training. The transcriptional activation of endothelial genes was also influenced by the interaction.

There was little overlap between genes altered by HIT and MOD. HIT was associated with pathways for inflammatory responses, while NON MOD genes showed enrichment for vessel growth pathways. In conclusion, the present findings provide strong evidence that genetic background influences endothelial function and its responses to exercise training. Several QTL/candidate genes are suggested as new targets for elucidating the genetic basis of intrinsic endothelial function. Exercise training has non-uniform effects on endothelial function and transcriptional activation of endothelial genes depending on the interaction between genetic background and training intensity.

DEDICATION

This work is dedicated to my lovely parents, Dongjin Kim and Insook Song,

for their limitless love and unconditional support. It's a blessing for me to be your son.

ACKNOWLEDGEMENTS

First and foremost, I would like to acknowledge my parents, Dongjin Kim and Insook Song, and my older sister Namin Kim. None of this would have been possible without your love and patience. You have been a constant source of love, concern, support and strength all these years. I'm so proud of being your son and brother.

I would like to thank my master's advisor, Dr. Sungjin Yoon, for making the field of exercise physiology so interesting and encouraging me to pursue Ph.D degree in USA. Thank you to my seniors, Drs. Charles Youngmok Jang and Moonhyon Hwang, for your support and advice. You both were my brilliant mentors.

I thank my former and current lab-mates, Dr. Sean Courtney and soon-to-be Ph.D Josh Avila, for your support, help, and opinions from different angles. You both were good examples of hard work that showed me how to become a better researcher. I want to express special thanks to Josh for having been not only a great lab-mate, but also a good friend. You are the one with whom I've spent the most of time here. Past 6 years sharing with you will be unforgettable memories in my life.

I thank Dr. Woodman and his past/current lab members. Dr. Woodman was always there to answer any questions I had regarding vascular biology. Your advice helped me develop my project in the right direction and your attitude of enjoying the research motivated me to become an active researcher like you. I thank Dr. Dan Trott for sharing your time with me to discuss research. I also thank John for being a great office mate. You and Josh have made the office a fun place to be.

Thank you to Dr. Lightfoot. I appreciate your valuable input in many aspects of these projects, for the time and interest you have taken in furthering my career. Your advice has helped me to think outside the box. The grant writing class you taught will always be my favorite. Thank you to his lab members, Dr. Emily Schmitt, Heather Vellers and Blaise Collins, for your kindness, generosity and thoughtfulness.

I also thank Dr. Penny Riggs for being generous with your time and input for this project. I'm grateful to have had the opportunity to do some work in your lab. This was great opportunity to learn and allowed me to expand my experimental skills. I thank Ms. Kelli Kochan for the time you spared for me to teach gene expression analysis.

Thanks also go to my colleagues, the department faculty and staff for making my time at Texas A&M University a great experience. My time here was made enjoyable due to many friends that became a part of my life. Time I spent with Drs. Jaewon Park, Yunmi Park and Minjung Kim is the most valuable memories I had from College Station. I would also like to thank AKOZ basketball team members and Korea University Alumni members at Texas A&M University for being my friends.

Most importantly, none of these was possible without my advisor, Dr. Massett. I would like to express my heartfelt gratitude to you for all your contributions of time, guidance, and funding to make my Ph.D experience productive and stimulating. You provided me with an excellent atmosphere to be an independent thinker. You've shown me an ideal role model as both a researcher and educator. I also want to express special thanks to Dr. Villalobos for support and generosity. I hope that we will be able to keep in touch for many years.

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1. INTRODUCTION

1.1. Clinical relevance

Almost one-fourth of Americans have some form of cardiovascular disease (CVD), which is responsible for more than six million hospitalizations and accounts for up to 40 % of deaths (196). Moreover, prevalence of CVD is increasing at younger ages (227). Epidemiological studies demonstrated that endothelial function can be a predictor of future cardiovascular health problems (131, 223, 288). Impaired endothelial function has been considered one of the fundamental components of hypertension and atherosclerosis (38, 54, 105, 108, 242, 269). The endothelium plays an important role in the regulation of vasomotor tone and the maintenance of vascular integrity (2, 55, 70, 158, 270).

Among several environmental factors known to influence endothelial function, exercise has been particularly highlighted for the last decade because regular exercise can prevent, as well as correct and/or improve impaired endothelial function (97, 146, 158, 239, 277). However, accumulating data indicate that there is considerable inter-individual variation in responses of the cardiovascular system to exercise, including changes in endothelial function (25, 96, 212, 245). Although environmental factors contribute to some of this variation, understanding the contribution of genetic background to endothelial function and its responses to exercise is an important research agenda. Outcomes from this study can help clarify the pathological mechanism/pathway

of endothelial dysfunction, thus provide the potential to enhance prediction of disease risk and the therapeutic targets for treatment of CVD related to endothelial dysfunction.

1.2. Endothelial function

The term endothelium was first used by Wilhelm His, a Swiss anatomist, in 1865 to define cells lining blood vessels and the mesothelial-lined body cavities (2). Since then, advancements in electron microscopy in the 1960s and cell biology in the 1980s enabled researchers to characterize the endothelium more precisely (82, 135). Currently, the endothelium is defined as the innermost cellular layer of blood vessels. Since the 1980s when endothelium-derived relaxing factors were discovered (90), the endothelium has been widely studied as an important modulator of vascular function. Its location in the internal lumen of blood vessel allows the endothelium to sense changes in hemodynamic forces and blood-borne signals and then respond by releasing vasoactive molecules (2, 153, 269, 270). The molecules released from endothelium have many physiological functions in maintaining vascular integrity: vasomotor tone control, cellular adhesion, thromboresistance, smooth muscle cell proliferation, permeability, and vessel wall inflammation (2, 105, 158, 218, 269, 270, 278). For example, vasomotor tone is controlled by vasodilators and vasoconstrictors produced from the endothelium (2, 158, 270, 278). Cytokines and adhesion molecules released from the endothelium regulate cell adhesion, permeability and proliferation (105, 218). The endothelium also releases molecules that regulate platelet activity, clotting cascade and the fibrinolytic system in response to inflammatory signals (258, 269, 278). Endothelial dysfunction

refers to deleterious alteration in production and bioavailability of those molecules, exhibiting features such as a decrease in endothelium-dependent vasodilation, an increase in adhesion and inflammatory molecules and an increase in oxidative stress (35, 70, 105). Given the important roles of endothelium in maintaining vascular health, the assessment of endothelial function has served as a useful biomarker of CVD.

1.2.1. Endothelium-derived vasoactive factors

Endothelial function is often represented by the ability of blood vessels to dilate via endothelium-dependent processes (55, 152, 269). Three molecules, nitric oxide (NO), prostacyclin (PGI_2) and endothelium-derived hyperpolarizing factors (EDHFs), have been considered among the most important dilator molecules. These molecules are released from endothelial cells as a common result of increased intracellular calcium (Ca^{++}) in response to mechanical signals, e.g. shear stress, caused by blood flow against the vessel wall or increased hormones/molecules that act through receptors (55, 65, 158, 269). Most importantly, a primary role of NO in vasodilation, as well as atheroprotective and thromboresistant influences, has been widely recognized in the literature (2, 158, 269, 270, 278). NO is synthesized in endothelial cells from L-arginine in a reaction catalyzed by endothelial nitric oxide synthase (eNOS), whose activity is regulated by intracellular Ca^{++} concentration (157, 158, 192). NO diffuses freely into vascular smooth muscle cells and binds to soluble guanylate cyclase (sGC), subsequently elevating cyclic guanosine monophosphate (cGMP). In turn, cGMP-dependent kinase is activated and intracellular proteins, such as myosin light chain kinase (MLCK) and Ca^{++}-activated

potassium channels, are phosphorylated. Consequently, smooth muscle relaxes via lowering intracellular Ca^{++} or desensitizing the muscle to Ca^{++} (77, 86). Previous studies have clearly shown that CVD is associated with reduced NO generation or bioavailability (54, 105).

PGI_2, which acts independently of NO (55, 65, 188), is synthesized via more complicated steps compared with NO. Phospholipase A_2, cyclooxygenase (COX) and prostacyclin synthase are serially involved in PGI_2 synthesis in endothelial cells (65, 158, 188). Increased PGI_2 results in G-protein-mediated activation of adenylate cyclase leading to the formation of cyclic adenosine monophosphate (cAMP) from adenosine triphosphate. cAMP ultimately reduces Ca^{++} in vascular smooth muscle cells, resulting in vascular smooth muscle relaxation (55, 65, 158, 188). Previous studies reported that a decrease in PGI_2 abolishes endothelium-dependent dilation (281) and accelerates CVD (7). As such, PGI_2 appears to have a role in endothelial function regulation, however fewer studies have examined the role of PGI_2 in the regulation of vasodilator tone in humans compared with NO (55, 65).

Endothelium-dependent dilation cannot be fully accounted for by NO and PGI_2, suggesting the existence of undefined endothelium-dependent vasodilating pathways. For example, after double gene-disruption of eNOS and COX-1, endothelium-dependent vasodilation persisted in mesenteric arteries from female mice (238). This finding supports the notion that NO- and PGI_2-independent vasorelaxation mediators exist. These independent mediators inducing smooth muscle hyperpolarization via NO- and PGI_2-independent pathways are called EDHFs. This role of EDHFs in mediating

endothelium-dependent relaxation appears particularly important as a compensatory mechanism when NO bioavalilabiltiy is reduced (47, 55, 77, 107, 112, 125, 126). Several substances have been proposed as putative EDHFs, e.g. epoxyeicosatrienoic acids derived from cytochrome P450 (CYP), lipoxygenase (12-(s)-hydroxyeicosatetraenoic acid (12-S-HETE), potassium ions (K^+) and vasoactive peptides (47, 76, 77, 99). One recently proposed EDHF candidate is hydrogen sulfide (H_2S), which induces vasodilation via stimulating ATP-sensitive K^+ channels in vascular smooth muscle (166, 291). In mice lacking cystathionie γ –lyase (CSE), an enzyme synthesizing H_2S, endothelium-dependent vasodilation was impaired (286). By contrast, the inhibition of CYP 2C9 contradictorily enhanced endothelium-dependent vasodilation in coronary disease patients (78). The authors noted that CYP 2C also generated O_2^-, thus blocking of CYP 2C might increase NO bioavailability. As described above, the identity and role of EDHFs are still controversial (76, 77, 188), and remain to be elucidated.

The endothelium produces not only vasodilators, but also vasoconstrictors, such as endothelin-1 (ET-1) and prostanoids (2, 55, 158, 269). An imbalance between vasodilators and vasoconstrictors could be a characteristic of endothelial dysfunction. In healthy endothelium, NO is preserved and suppresses ET-1 production. Verhaar et al. reported that increased forearm vasodilation induced by ET-1 receptor antagonist (BQ123) was reversed by NO inhibitor (L-NMMA) in healthy subjects (268). However, under the condition of impaired endothelial function, ET-1 expression is increased and ET-1 may decrease eNOS expression, thereby vasoconstriction becomes exaggerated

(26, 165). This is further supported by other findings that administration of ET-1 impairs endothelium-dependent dilation in healthy individuals, while ET-1 receptor antagonists increased vessel diameters and blood flow in CVD patients (29). Increased ET-1-mediated vasoconstriction has been linked to a number of cardiovascular pathologies, such as hypertension, vasospasm and coronary artery disease (190, 263). Angiotensin-converting enzyme (ACE) inhibition also improved endothelium-dependent vasodilation via increases in NO, PGI and EDHFs in responses to bradykinin (191, 221). Therefore, the balance between the vasodilators and vasoconstrictors released from endothelium is critical for vascular health.

1.2.2. Assessment of endothelium-dependent dilation

Endothelium-dependent dilation has been assessed both *in vivo* and *in vitro* in response to mechanical signals or vasodilating agents (97, 115, 158, 184, 269). There are several noninvasive measurements in human subjects, e.g. doppler echocardiography, positron emission tomography and phase-contrast magnetic resonance imaging, to assess *in vivo* endothelial function during increased blood flow (80, 105). The most commonly used noninvasive assessment is flow-mediated dilation (FMD) in the brachial artery. FMD measures vasodilation induced by reactive hyperemia after release of acute occlusion of the brachial artery. This acute increase in blood flow exerts shear forces to the vessel which stimulate endothelial cells to release NO, PGI_2 and EDHF and, in turn, relaxes vascular smooth muscle (55, 65, 115, 153, 282). Several invasive assessments have been also utilized to measure endothelial function in response to intra-arterial or

intravenous infusion of endothelium-dependent vasodilators, such as acetylcholine (ACh), bradykinin and substance P (80).

In animal models, endothelial function is primarily assessed by measuring responses of isolated vessels to vasoactive molecules or flow-induced shear forces *in vitro* (58, 111, 119, 136, 152, 184). A wire myograph system is generally used to assess vasomotor function in large and small conduit vessels. This system allows investigators to examine vasomotor function (change in isometric tension) of isolated vessels under various physiological conditions (184). Alternatively, vascular function of small arteries and resistance vessels can be measured using perfusion with micropipettes linked to pressure reservoirs. This experimental setup enables investigators to measure flow-induced dilation with adjustments of pressure gradient under certain physiological conditions (136, 152). In isolated vessels prepared by these experimental setups, the application of endothelium-dependent vasodilators with presence or absence of pharmacological agonists/antagonists enables the measurement of endothelium-dependent, as well as pathway-specific vasomotor regulation. For example, comparing ACh-induced vasorelaxation in vessels treated with eNOS inhibitor (e.g. L-NMMA or L-NAME) or vehicle provided evidence that ACh-induced vasorelaxation is NO-dependent (111, 119, 281). In contrast, the application of sodium nitroprusside (SNP), a NO-donor that elicits endothelium-independent vasodilation, has been commonly utilized to assess smooth muscle relaxation function (58, 142, 281).

The changes in diameter or in isometric tension induced by vasoactive agents or shear force can be compared with baseline diameter or tension. Vasomotor function is

generally expressed as a percentage for the comparison, such as % FMD or % vasorelaxation. There is an abundance of evidence showing reduced % FMD in patients suffering CVD, e.g. hypertension, atherosclerosis, diabetes, coronary artery diseases and heart failure (35, 70, 105, 242, 278) compared to healthy individuals and decreased % vasorelaxation in response to endothelium-dependent agonists in vessels isolated from animal models of CVD (42, 163, 195).

1.3. Effect of exercise training on endothelial function

Regular exercise has long been considered necessary for maintaining cardiovascular health. Improvement in physical fitness via regular exercise is inversely related to all-cause and cardiovascular mortality (21, 161). A vast majority of previous studies have provided overt evidence that exercise yields many beneficial effects on CVD risk factors, such as weight loss, lowering blood pressure, higher insulin sensitivity and lowering lipids (147, 241). It has been also well established that regular exercise exerts beneficial effects on endothelial function. In particular, clinical studies have demonstrated that regular exercise reverses endothelial dysfunction in CVD patients, e.g. heart failure (111), hypertension (119) and diabetes (88). Chronic exercise has also been shown to improve endothelial function in young healthy subjects (46, 59, 119). In animals, exercise training improved endothelial function in both healthy animals and disease models as well (58, 137, 145, 158).

Two possible mechanisms exist for the beneficial effects of chronic exercise on endothelial function: hemodynamics effects (shear stress) and risk factor modification

(153, 261). Select studies have supported the hypothesis that changes in circulating molecules, e.g. hormones, cytokines, adipokines, contribute to the systemic benefits of exercise training on endothelial function (40, 163, 204). For example, Lee et al. reported that exercise training improved endothelial function in diabetic mice through both adiponectin-dependent and independent pathways (163). However, in many cases, exercise training improved endothelium-dependent dilation without major changes in CVD risk factors (46, 97, 98), implying that regular exposure to increased shear stress might be the primary signal for exercise training induced-adaptations of endothelial function. Alterations in circulating molecules would, therefore, be systemically secondary effects (153, 261). Shear stress, particularly laminar shear stress, during exercise is known to increase anti-atherogenic and decrease pro-atherogenic endothelial cell phenotypes, e.g. increases in eNOS and superoxide dismutase (SOD) *vs.* decreases in cell adhesion molecules (153, 218, 285). Chronic exposures to such effects of shear stress via regular exercise would promote beneficial adaptations in endothelial function.

1.3.1. Effect of exercise training on nitric oxide pathway

Results from both human and animal studies have demonstrated that exercise training enhances endothelial function (57, 58, 110, 119, 194, 240). Following a standardized 12-week exercise program, forearm blood flow in response to ACh infusion was increased significantly in both normotensive and hypertensive adults (119). Infusion of an eNOS inhibitor (L-NMMA) blunted the training-induced increases in forearm blood flow in response to ACh, indicating that training-induced increases were mediated

by NO. Hambrecht and colleagues also observed that regular exercise for 4 weeks increased vessel diameter and peak blood flow velocity in response to ACh in coronary artery disease patients (110). Exercise trained-patients had higher level of eNOS gene/protein expression and phosphorylation of eNOS protein compared with non-trained patients. These findings are in agreement with data from animal studies. Graham and Rush found that exercise training enhanced vasorelaxation responses to ACh in aortic rings from spontaneously hypertensive rats (94). The training-induced enhancement in vasorelaxation to ACh was abolished in the presence of nitric oxide synthase inhibitor (L-NAME), indicating that training-induced enhancements were dependent on NO pathway. Sessa et al. reported that the underlying mechanism for improved endothelium-dependent dilation by exercise training is an increase in eNOS gene expression and subsequent NO production (240). In another study, eNOS protein levels were also increased by exercise training with enhanced vasodilator responses to ACh (57). Combined, those findings indicate that exercise training enhances endothelial function via increasing eNOS expression and NO production. In contrast, physical inactivity induced by hindlimb unloading (2 weeks) impaired endothelium-dependent dilation in addition to lowering eNOS gene and protein expression in rat soleus arterioles (236).

The amount and bioavailability of NO are determined by not only the activity of eNOS, but also NO-scavenging mechanisms, such as the reaction with superoxide (O_2^-). In presence of O_2^-, NO readily reacts with O_2^- to form peroxynitrite (ONOO-) with high affinity. Accordingly, an increase in O_2^- via disrupted endogenous antioxidant system

results in NO degradation and consequently a net reduction in NO bioavailability (35, 146, 201). Moreover, ONOO- produced by the reaction of NO with O_2^- is known to damage a wide array of molecules in cells and contribute to the pathogenic mechanisms of CVD, including endothelial dysfunction (201). It has been shown that increased O_2^- production accounts for decreased NO bioavailability in CVD patients and animal models of CVD (35, 146, 183). The administration of superoxide dismutase (SOD) mimetics, e.g. tempol and apocynin, reversed impaired endothelium-dependent vasodilation in animal models of CVD (215, 289). In contrast, cumulative bouts of exercise can upregulate antioxidant enzymes, such as SOD, catalase and glutathione peroxidase (GPx), which scavenge free radicals. Exercise training can also downregulate nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and uncoupled eNOS, which are sources of O_2^- (72, 89, 146, 232, 239). Previous data indicate the beneficial effects of exercise training on NO bioavailability via both increasing free radical scavengers and decreasing oxidative stress molecule production.

1.3.2. Effect of exercise training on other vasodilators, vasoconstrictors and vascular smooth muscle

PGI_2 and EDHFs are also important mediators for exercise training-induced improvements in endothelial function (91, 137, 148, 158, 277). Koller et al. examined the effect of 3-week exercise training on endothelium-dependent vasodilation in isolated gracilis muscle arterioles of young rats (148). They found that exercise training enhanced endothelium-dependent vasodilation and this enhanced vasodilation was

reduced by both eNOS inhibitor (L-NMMA) and COX inhibitor (indomethacin) by similar amounts (40 to 50 %). Muller and colleagues reported a similar finding that training-induced improvements in endothelium-dependent vasodilation to bradykinin were abolished by either indomethacin or L-NMMA (194), demonstrating that training-induced augmentation of endothelial function is due to increases in both NO and PGI_2. In young healthy individuals, Zoladz and colleagues found that an acute bout of exercise increased PGI_2, as assessed by plasma 6-keto $PGF_{1\alpha}$ concentration (294). In a follow-up study, the authors found that 5-week endurance training augmented the release of PGI_2 in responses to exercise (295). These data are in accord with results observed from hypertensive subjects (112), demonstrating that exercise training enhances endothelial function partially via enhancing PGI_2 production.

Woodman and colleagues reported that enhanced endothelium-dependent dilation in brachial arteries from hypercholesterolemic pigs after 16-week endurance exercise training persisted in the presence of both eNOS and COX inhibitors (L-NAME and indomethacin. respectively (283). Their finding indicated that enhancements in endothelium-dependent dilation after exercise training were due to, in part, enhanced production of EDHF. In hypertensive rat models, neither eNOS inhibitor nor COX inhibitor blocked training-induced improvements in endothelium-dependent dilation in muscle feed arteries (104). In contrast, a potassium (K^+) channel blocker (tetraethylamonium) abolished the training-induced improvements in endothelium-dependent dilation, indicating that exercise training improves endothelial function partly via EDHF pathway, particularly in CVD animal models. Increased plasma and aorta H_2S

levels accompanied by improved endothelium-dependent relaxation were observed after exercise training in young hypertensive rats (102). By contrast, Hansen et al. observed no changes in expression levels of CYP2C9, CYP4A and CSE after exercise training in muscle samples of hypertensive subjects (112). To date, the role of EDHFs in mediating endothelial responses to exercise training is still a controversial field of study.

Exercise training also alters responses to vasoconstrictor agents (42, 58, 138, 176, 209, 210, 248). For example, decreased vasocontractile responses to ET-1, Angiotensin II (ANG II), norepinephrine (NE) have been reported after exercise training (58, 138, 176, 209). For ET-1, 8-week exercise training substantially decreased plasma level of ET-1 which remained depressed for 4 weeks after the cessation of exercise training in healthy humans (176). [^{32}P]phosphatidic acid, an indicator of phospholipase activity induced by ET-1, was also reduced in pig coronary artery after exercise training for 16 to 20 weeks (138). In general, attenuated vasoconstriction is associated with improved endothelial function. For instance, Park and colleagues found that exercise training (10 to 12 weeks) attenuated the ANG II-induced vasoconstriction in old rats (209). However, this attenuated vasoconstriction after exercise training was abolished by endothelium removal or eNOS inhibition, demonstrating that training-induced attenuation of vasoconstriction is mediated by NO pathway. This is also supported by Maeda and colleagues who found that decreased plasma levels of ET-1 after exercise training was accompanied by increased plasma NOx levels (176).

During exercise, vascular smooth muscle cells not only receive molecular signals from endothelium, but are also exposed to transmural pressure and cyclic strain

generated by increased blood flow. These flow-induced mechanical forces might induce changes in mechanotransduction in smooth muscle cells *per se*. Notwithstanding, there are only a handful of studies that investigated vascular smooth muscle adaptations to exercise training (27, 28, 116, 271). For example, 12 weeks of exercise training decreased Ca^{++} release from sarcoplasmic reticulum (SR) in response to ET-1 in porcine coronary arteries (28, 271). The authors noted that this training-induced decline in Ca^{++} release might account for the observations that exercise training reduces contractile responses to vasoconstrictors. Jones et al. reported that training-induced reduction in contractile responses to ET-1 in male swine coronary arteries was reversed by blocking K^+ channels (138). These findings support the hypothesis that exercise training might alter smooth muscle function via, in part, by reducing intracellular Ca^{++} regulation and increasing K^+ channel activity. In contrast, vascular smooth muscle relaxation response to SNP is not generally changed by exercise training (58, 114, 144, 283).

1.4. Intensity-dependent effect of exercise training on endothelial function

Although regular exercise usually yields favorable effects on cardiovascular health, the exercise components, such as intensity, duration and frequency, required to establish the optimal training strategies are still debated. In studies of direct comparisons of exercise intensity and duration (162, 259), the exercise intensity was associated with reduced CHD risk independent of the total volume/duration of exercise. Even in subjects who did not perform vigorous exercise regularly, walking pace was also associated with reduced CHD risk independent of the amount of walking hours (259). Previous findings

suggest that exercise intensity might have a more prominent effect on CHD prevention than duration or volume of exercise.

1.4.1. Training intensity and cardiovascular traits

High intensity interval training (HIT), characterized by intermittent bursts of vigorous activity interspersed by periods of rest or active rest, has been proposed as an effective alternative to traditional endurance training on a matched-work basis or equivalent estimated energy expenditure. For a wide range of physiological and health-related markers in both patient and healthy populations, HIT exerted similar or even superior effects compared with moderate intensity continuous training (MOD) (175, 181, 262, 265). Swain et al. noted in their cross-sectional study that vigorous intensity (typically ≥ 60% VO_2max) exercise training generally exerts greater cardioprotective benefits compared with moderate intensity exercise training when total work is equated (257). Animal studies also reported greater effects on cardiovascular traits, such as VO_2max and blood pressure, after HIT (typically 75 to 90 % of VO_2max) compared with MOD (typically < ~70% of VO_2max) (129, 144). However, several studies have provided conflicting data, e.g. similar effects between HIT and MOD (106, 228), no effect of HIT (45), and even potential adverse effects of HIT (12, 121, 273) on cardiovascular traits in various populations. Indeed, HIT would not be safe, tolerable and applicable for some populations, e.g. elderly and patients. Therefore, the optimal training intensity for maintaining/improving cardiovascular health has yet to be determined.

1.4.2. Effect of training intensity on endothelial adaptation to exercise training

Over the last decade, the effect of training intensity on endothelial function has been examined in both humans and animals. The majority of previous studies in CVD patients have exhibited greater improvements in endothelial function in response to HIT compared with MOD (189, 262, 280). These superior effects of HIT on endothelial function were accompanied by greater improvements in VO_2max, antioxidant status and blood metabolites. Greater effects of HIT on endothelial function in CVD patients could be due to higher blood flow in HIT leading to greater shear stress-induced NO production and an increase in NO bioavailability induced by an increase in antioxidant status. On the contrary, the effect of training intensity on endothelial function is more complicated in young healthy individuals. In a study conducted by Rakobowchuk and colleagues, both HIT and MOD improved endothelial function to a similar extent in young individuals (222). In contrast, Goto et al. reported that endothelial function was improved by MOD, but not by HIT, in young subjects (93). An increase in oxidative stress was found in the HIT-trained subjects. The authors speculated that the absence of endothelial function change in HIT might be due to HIT-induced increases in oxidative stress which reduced the bioavailability of NO increased by HIT. This is further supported by a study conducted by Bergholm and colleagues who found declines in endothelial function and circulating antioxidants after 3 months of intense exercise training (70 to 80 % VO_2max) (16). Those findings raise the possibility that vigorous exercise could yield negative effects on endothelial function via increasing oxidative stress in young healthy individuals.

Endothelial responses to different training intensities are also inconsistent in animal studies. Haram et al. reported that HIT enhanced endothelium-dependent dilation more than MOD in rat abdominal aortas (114), while Kemi et al. found that both HIT and MOD improved endothelium-dependent dilation to the same magnitude in rat carotid arteries (144). Both studies showed greater improvements in VO_2max after HIT compared with MOD. Such discrepant results in both humans and animals might be ascribed to heterogeneity in baseline health, age, sex, training duration, timing of measurement, and vascular bed. Those factors have been known to influence the effect of exercise training on endothelial function (96, 97, 137, 146, 158, 277). Additional studies that minimize those environmental factors are needed to compare the effect of training intensity solely on endothelial function.

1.5. Genetic regulation of endothelial function

Cardiovascular traits are regulated by not only environmental factors, but also genetic factors and/or the interaction between environmental and genetic factors (19, 83, 187). For instance, Mitchell and colleagues reported that environmental covariates and genetic factors accounted for <15 % and 30 to 40 % of variation in plasma lipid profiles in Mexican Americans, respectively (187). In an epidemiological survey, genetic factors accounted for about 30% of blood pressure variation observed in a large cohort of participants (19). These findings demonstrate that the cardiovascular traits are partly regulated by genetic factors. For endothelial function, several studies in both humans and animals have provided evidence for genetic regulation of this trait. Candidate gene

studies have revealed that polymorphisms in a few endothelial genes are associated with endothelial function in humans (36, 71, 85, 132, 139, 208, 230). The estimated heritability (0.14 to 0.44) of endothelial function has been reported in various populations (13, 122, 255, 267, 290) and a racial-dependent difference was observed for endothelial function (179, 200). A human genome-wide association study (GWAS) also provided limited data regarding single nucleotide polymorphisms associated with FMD (267). Previous studies demonstrate that endothelial function is a polygenic, heritable trait.

1.5.1. Evidences from human studies

Experimental and clinical studies suggest that genetic variation in eNOS can influence endothelial function. To date, more than 100 polymorphisms have been identified in the NOS3 (eNOS) gene. Among many, two polymorphisms ($T^{-786}\rightarrow C$ and $G^{894}\rightarrow T$) are the most studied NOS3 gene polymorphisms (36, 64, 132, 139, 208, 230). $T^{-786}\rightarrow C$ resides in the promoter region where it regulates transcriptional initiation of NOS3 (139). Endothelial cells from coronary heart patients carrying CC genotype at $T^{-786}\rightarrow C$ exhibited a reduction in NOS3 mRNA and protein expression in response to laminar shear stress compared to those from patients carrying the 'T' allele (36). Similarly, hypertensive subjects carrying CC genotype showed lower vasodilatory responses to ACh compared with subjects carrying TT genotype (230). $G^{894}\rightarrow T$ polymorphism maps to exon 7, resulting in replacement of glutamate to aspartate at codon 298 (also denoted as Glu298Asp) (139). Ingelsson and colleagues found that

G^{894}→T was significantly associated with FMD, showing that TT genotype carriers had higher FMD compared with GG or GT genotype carriers (132). Similarly, plasma NOx concentration in subjects having GG genotype was relatively lower than subjects carrying the 'T' allele (64). However, these results are not consistent. Ingelsson and colleagues did not find the polymorphic effect of T^{-786}→C on endothelial function (132). Paradossi et al. also reported that the polymorphism of T^{-786}→C was not associated with endothelium-dependent vasodilation. Furthermore, subjects carrying TT genotype at G^{894}→T showed lower endothelium-dependent vasodilation (208). Thus, the effects of these polymorphisms on endothelial function are variable and might depend on the study population or other genetic factors.

Polymorphisms of genes associated with NO bioavailability have also been tested for associations with endothelial function. Genetic variation in p22phox subunit of NADPH oxidase (CYBA) gene, which produces O_2^-, has been studied as a factor influencing NO bioavailability (71, 85, 139, 235). Among several polymorphisms of this gene, C^{242}→T has been the primary focus (71). For the C^{242}→T polymorphism located in exon 4, substituting histidine to tyrosine, Fan and colleagues found that 'T' allele carriers showed relatively higher brachial FMD (%) than 'C' allele carriers in young individuals (71). Fricker et al. also provided similar observations that TT carriers had greater vasodilatory responses to bradykinin compared with other genotypes in healthy men (85). However, the polymorphic effect of C^{242}→T on endothelial function is not always significant (235). For other endothelial genes, e.g. 6R-tetrahydrobiopterin (BH_4), asymmetric dimethylarginine (ADMA), and ACE, the associations between their

polymorphisms and endothelial function are discordant in the literature as well (139). Indeed, there is still lack of a reliable molecular marker, such as NO or O_2^- concentration, possibly due to technical complexity. Although previous data have emphasized a few genes as important genetic determinants of endothelial function, the reliable association of each polymorphism with endothelial function has not been firmly drawn so far.

Vasan and colleagues assessed various cardiovascular traits, including FMD (%), in 1,345 subjects from the community-based Framingham Heart Study (https://www.framinghamheartstudy.org/) (267). The authors conducted GWAS for those traits using a 100k single nucleotide polymorphism set, and as a result, identified several single nucleotide polymorphisms associated with each cardiovascular trait. In the case of FMD (%), the peak single nucleotide polymorphism (p = 1.13e-05) was found on human chromosome 7. This study was the first to conduct a GWAS approach directly for endothelial function in a large size sample population, offering a fundamental framework for GWAS of endothelial function. However, data provided in this study had limited impact on the identification of causal candidate genes responsible for FMD because none of associations was reached at the significant level suggested for human GWAS (5×10^{-8} to 10^{-7}) (207). Furthermore, their findings have not been replicated.

The population structure and sample size have been major considerations regarding statistical power of GWAS in human studies (48, 141). Together with the possibilities of phenotyping errors and environmental influences, those obstacles have hindered segregation of the genetic influence *per se* and replication of findings in human studies. Accordingly, animal models have been alternatively utilized for genetic

association studies due to the advantages provided by a minimal environmental influence, genetic homozygosity, and accessibility of disease-relevant tissues (33, 81).

1.5.2. Evidence from animal studies

Candidate gene studies have been often conducted with genetically modified animals. In particular, gene knockout mouse models have been commonly utilized, allowing investigators to test a direct functional role of a gene in phenotypes or diseases and offer a biological context facilitating investigation of associated-signaling pathways (5, 10, 109). Many efforts have utilized knockout mouse models of endothelial genes to elucidate the genetic basis for endothelial function. For example, eNOS knockout mice showed impaired endothelium-dependent vasodilation compared with wild-type mice (127). eNOS knockout mice have also manifested pro-atherogenic phenotypes, e.g. increased platelet aggregation, leukocyte adhesion, and propensity to thrombosis (8). In contrast, mice overexpressing eNOS had elevated eNOS activity and net NO levels, thus NO bioavailability, in aortas compared with wild-type mice (11). Significant impairments in endothelium-dependent vasodilation observed in CSE gene knockout mice and CuZnSOD-deficient mice also confirmed their important roles in endothelial function as an EDHF and a superoxide scavenger, respectively (61, 286). However, limitations exist in generating knockout mice, for instance, developmental lethality, difficulties in knocking out certain genes/loci, and changes in unrelated phenotypes (109). Accordingly, gene-specific knockout mouse studies have not yielded as many valid genes and their targets as anticipated.

An alternative genetic approach to the usage of genetically modified mice is an inter-strain comparison of a phenotype of interest across different inbred mouse strains. The phenotypic diversities across inbred mouse strains make it possible to identify novel gene(s) responsible for the phenotype via the association analysis between phenotype and genotype over the entire mouse genome (5, 79, 81). Previously, vasoreactivity in isolated aortas was assessed and compared among several inbred mouse strains (41, 233). In both studies, there were strain-dependent differences in endothelium-dependent vasorealxation among inbred mice. Ryan and colleagues found that 129-substrains of mice had markedly reduced aortic responses to ACh compared with 5 other inbred strains of mice (233). In the study conducted by Chen et al., aortas from SJL/J mice had lower vasorelaxation responses to ACh (~40%) than aortas from C3H/HeJ and FVB/NJ inbred mice (41). Parallel to lower responses to ACh, SJL had decreased eNOS and SOD-2 protein expression, implying that decreases in SOD-2 and eNOS level may contribute to impaired vasorelaxation responses to ACh in inbred SJL. Those findings provide evidence that genetic background influences endothelial function in mice.

Supportive corroborations have been further offered by investigations in genetically manipulated rats. Selectively bred rats up to 11 generations for low aerobic capacity exhibited relatively lower ACh-induced vasorelaxation in carotid arteries compared with rats selectively bred for high aerobic capacity (279). A consomic rat panel was created based on normotensive Brown Norway (BN) and Dahl salt sensitive (SS) inbred rat strains by substituting BN chromosomes onto SS inbred rat (http://pga.mcw.edu/) (51). Comparison of a phenotype in consomic rat strains with

parental SS inbred strain affords the opportunity to discover chromosomes that may contain genes contributing to the phenotype. Using the consomic rat panel, Kunert and colleagues assessed vasoreactivity in isolated aortas (150). The authors found that aortic rings from consomic rat strains of chromosomes 16 and Y had greater sensitivity to ACh, while aortic rings from consomic rat strains of chromosomes 9, 13 and 20 had reduced sensitivity to ACh, compared with parental SS inbred strain. These results indicate that chromosomes 9, 13, 16, 20, and Y contain genetic factor(s) responsible for sensitivity to ACh. In a separate study, the same group of investigators conducted similar experiments, but in a different consomic rat panel constructed from BN and Fawn Hooded Hypertensive (FHH) rat strains (151). Consomic rat strains of chromosomes 3, 4, 5, 10, 11, 12, 14, and Y had different sensitivity to ACh compared to parental FHH inbred rats. However, only one chromosome (Y) overlapped between two studies, implying that chromosomes responsible for endothelial sensitivity to ACh are strain-specific in rats (150, 151). Collectively, previous findings in animal studies clearly indicate that endothelial function has genetic regulation, eliciting the necessity of comprehensive genomic scans via objective and unbiased hypothesis-free tests to specify the genomic loci responsible for regulating endothelial function.

1.5.3. Genetic regulation of vascular smooth muscle function

Limited evidence indicates that vascular smooth muscle function is influenced by genetic factors. In the aforementioned studies regarding mouse strain comparison for vasoreactivity (41, 233), endothelium-independent responses to SNP (NO donor) were

different at low doses (10^{-9} to 10^{-7} M) among inbred mouse strains. These differences were not observed at higher doses (> 10^{-7} M) of SNP, suggesting that sensitivity, rather than maximal relaxing ability, of smooth muscle to NO is modified by genetic background. This was in line with studies that utilized a consomic rat panel. Kunert et al. found that aortic sensitivity to SNP in aortas from chromosome 16 consomic strain and BN inbred strains differed from SS parental inbred strain (150). In smooth muscle, NO acts mainly on NO-sensitive GC, which synthesizes cGMP inducing smooth muscle relaxation via activation of cGMP-dependent protein kinase, phosphodiesterases and ion channel gates. Friebe and colleagues found that aortic relaxation responses to NO donors were absent in GC-deleted mice (87). Wooldridge et al. generated knockout mice of smoothelin-like protein 1 (SMTNL1), a downstream effector of GC-mediated cGMP-dependent protein kinase (PKG), which is known to suppress myosin phosphatase activity in vascular smooth muscle (284). SMTNL1 knockout mice exhibited enhanced vasorelaxation responses to ACh without differences in eNOS protein expression and phosphorylation. Therefore, it can be speculated that genetic factors related to GC or its downstream effectors could contribute, in part, to vascular smooth muscle relaxation. This is further supported by Buys et al. who showed that $GC_{\alpha 1}$-deficient mice generated on a 129S6 background had significantly greater impairments in aortic vasorelaxation responses to ACh than $GC_{\alpha 1}$-deficient mice generated on a B6 background (34). Their findings further suggest that genetic background modulates the role of GC signaling in smooth muscle relaxation.

Smooth muscle contractile function may be affected by genetic background as well. A wide range of variation in dorsal hand vein responses to phenylephrine (PE; α_1-adrenergic agonist) was observed in a healthy adult population (217). In 44 individuals from 12 families, Gupta et al. reported the estimated heritability of 0.88 for α_1-adrenergic receptor responsiveness in superficial veins (103). These previous data support the notion that smooth muscle contractile function is a heritable trait. A candidate gene linking to smooth muscle contractile function has been proposed by Bergaya and colleagues (15). In that study, WNK lysine deficient protein kinase 1 (WNK1) gene haploinsufficient mice had markedly reduced vascular contractile responses to PE compared with wild-type mice without differences in relaxation response to ACh and contractile responses to potassium chloride (KCl). Their results suggest that WNK1 gene might be one of genes that play a role in smooth muscle contractile responses specific to α_1-adrenergic receptor activation. However, in the consomic rat panel, the sensitivity to PE was different in several consomic strains compared with parental SS inbred strain (150), indicating that smooth muscle contractile function, at least sensitivity to α_1-adrenergic agonist, might be influenced by multiple chromosomes. Nevertheless, only a few specific genes of interest have been examined, and thus the genetic basis for smooth muscle function is largely unknown.

1.6. Genetic contribution to endothelial responses to exercise training

It has become evident that the effect of exercise training differs substantially among individuals. The most distinguished data for individual variation in responses to

exercise training come from the HERITAGE Family Study (*HE*alth, *RI*sk factors, exercise *T*raining *A*nd *GE*netics) (http://www.pbrc.edu/heritage/) (23). This study has yielded identification of several gene polymorphisms associated with variation in responses of cardiovascular traits to exercise training via both candidate gene approach and genome-wide exploration (24, 25, 247, 260). However, endothelial function or FMD was not included in that study.

Recently, Green and colleagues published data showing a wide range of inter-individual variation in FMD (%) changes after exercise training (96). Among 182 subjects, 76 % exhibited improved FMD by exercise training, while 24 % showed no changes or even decreased FMD after exercise training. These findings illustrate that exercise training exerts non-uniform effects on endothelial function among individuals. An additional interesting result from this study was that changes in FMD after exercise training were not correlated with changes in traditional cardiovascular risk factors, such as VO_2max and mean arterial pressure, suggesting that the endothelial responses to exercise training are independent of changes in other cardiovascular traits.

Hopkins et al. examined the effect of exercise training on endothelial function in mono- and di-zygotic twins (6 pairs each) (123). Changes in FMD (%) after 8 weeks of aerobic exercise training were highly correlated in monozygotic twins (r=0.63), whereas changes in FMD (%) after exercise training were not significantly correlated in dizygotic twins. The estimated heritability of training-induced changes in FMD was 0.74 in this study. Feairheller and colleagues also found the racial difference in endothelial cell responses to exercise-mimicking shear stress (73). Human umbilical vein endothelial

cells (HUVECs) from African Americans had higher level NADPH oxidase subunit protein expression at baseline compared to Caucasians, while laminar shear stress modulated those protein expression to similar levels between race. The authors concluded that endothelial cells from African Americans might be more responsive to shear stress stimulus than those from Caucasians. Collectively, previous studies indicate that endothelial responses to exercise training are influenced by genetic background.

A handful of candidate gene studies examined the impact of genetic polymorphisms on endothelial responses to exercise training (6, 69, 198). In male coronary artery disease patients, supervised exercise training for 4 weeks at target heart rate (80% of maximal heart rate) improved ACh-induced average peak velocity (APV) in coronary arteries (69). This improvement varied by eNOS polymorphism. After exercise training, patients carrying 'C' allele at $T^{-786}\rightarrow C$ had a smaller improvement in APV (~ 36 %), compared with patients carrying 'T' allele (~ 81 %). A polymorphic effect of $G^{894}\rightarrow T$ was not observed in that study. Similarly, 18-week exercise training by young healthy males significantly increased forearm vascular conductance during handgrip exercise in TT carriers, but not in CT and CC carriers at $T^{-786}\rightarrow C$ of eNOS gene (198). Comparable endothelial responses to exercise training in healthy males were reported for polymorphism in type B_2 bradykinin receptors (B_2KR) gene (6). After exercise training, only -9/-9 carriers had increased forearm blood flow and vascular conductance during handgrip exercise. These training-induced increases were accompanied by a decrease in serum ACE enzyme levels. Like other cardiovascular traits, candidate gene studies, however, have employed only a few common genes for

their polymorphic effects on endothelial responses to exercise training. Furthermore, as discussed above, effects of exercise training on endothelial function are dependent on training intensity. It implies that there is a complicated interaction of genetic factors and training intensity on endothelial adaptation to exercise training.

1.7. Summary

The endothelium has a critical role in maintaining vascular integrity via synthesis of several vasoactive molecules. Accumulated data indicate that endothelial function is a heritable trait and is regulated by polygenic factors; however, these genetic factors have not been fully elucidated. Given the notion that single genetic variant generally has only small to modest functional effects, a large genomic scale analysis is necessary to comprehensively unravel the complex genetic basis of endothelial function.

Exercise training is well known to improve endothelial function. The effect of exercise training appears to be dependent on the training intensity; nevertheless, little is known about the effect of training intensity on endothelial responses to exercise training. Indeed, genetic contribution to endothelial responses to exercise training and its interaction with training intensity has not been formerly considered.

1.8. Purpose and hypotheses

Hence, the main purposes of this dissertation are 1) to identify quantitative trait loci (QTL)/candidate genes residing in the QTL responsible for intrinsic endothelial function and 2) to determine the interaction between genetic background and training

intensity on the endothelial adaptations to exercise training. To accomplish the purposes, two hypotheses were proposed:

1) Intrinsic endothelium-dependent vasorelaxation is largely variable across inbred mouse strains, and the variation is influenced by one or more quantitative trait loci.
2) Endothelial adaptations to exercise training are variable among inbred mouse strains and these variable adaptations are dependent on training intensity.

2. ASSOCIATION MAPPING OF GENETIC CONTRIBUTION TO ENDOTHELIAL FUNCTION IN MOUSE AORTA

2.1. Introduction

Almost one-fourth of Americans have some form of cardiovascular disease (CVD), which is responsible for more than six million hospitalizations and accounts for up to 40 % of deaths (196). There is strong evidence that susceptibility to CVD and related risk factors are highly heritable (67, 216, 266). Accordingly, numerous clinical and laboratory studies have strived to elucidate the genetic basis of CVDs, e.g. hypertension (52, 128, 133), atherosclerosis (120, 174, 252, 274), myocardial infarction (49, 197), and coronary artery disease (167, 226, 237).

The endothelium plays an important role in maintaining vascular integrity via release of various vasoactive mediators which control vasomotor tone, hemostatic balance, permeability, proliferation and survival (2, 270). Impaired endothelial function is a fundamental component of hypertension and atherosclerosis and hence, a predictive precursor for CVD (108, 131, 223). Many environmental factors, e.g. diet and physical activity level, are known to influence endothelial function (31, 137). Several lines of evidence from human studies demonstrated that endothelial function also has genetic regulation. Candidate gene studies have revealed that single nucleotide polymorphisms of some endothelial genes, e.g. endothelial nitric oxide synthase (*eNOS*) and p22phox subunit of NADPH oxidase (*CYBA*), are associated with flow-mediated dilation (FMD)

(71, 139, 143, 208), a non-invasive method for measuring endothelial function in humans. The estimated heritability of FMD has been reported in various human populations, ranging from 0.14 to 0.44 (13, 122, 255, 267, 290). Vasan and colleagues also conducted a GWAS to identify single nucleotide polymorphisms associated with brachial artery endothelial function traits in subjects from the Framingham Heart study (267). This GWAS identified one single nucleotide polymorphism (rs3814219, on chromosome 10) associated with baseline brachial artery flow velocity ($P < 1.00 \times 10^{-5}$). Previous studies indicate that endothelial function is a polygenic, heritable trait. However, the polymorphic effects of those genes on FMD were inconsistent (139, 143) and the previous GWAS provided only limited evidence with low statistical power.

Inconsistent results have often been observed in human genetic studies partially due to heterogeneity in population structure and inadequate sample size (48). Combined with phenotypic complexity and environmental influence, those potential limitations make the identification of actual genetic associations difficult and hinder the replication of findings in human studies. Alternatively, mice are being utilized in genetic studies. Mouse models have several advantages for genetic studies, e.g. a minimal environmental influence, the genetic homozygosity, and accessibility of disease-relevant tissues (81, 213). Therefore, a number of mouse linkage and association studies has identified quantitative trait loci (QTL) and/or candidate genes associated with CVD-related traits, e.g. atherosclerosis susceptibility (134, 250), blood lipids (160, 253), blood pressure (62, 74), heart rate (22, 246), and cardiorespiratory fitness (50, 180). A handful of rodent studies have reported the differences in endothelium-dependent vasorelaxation among

several inbred mouse strains (41, 233) and the effects of chromosome substitution on endothelium-dependent vasorelaxation in rat (150, 151), supporting the notion that endothelial function is regulated by genetic background. In spite of such successes from mouse models used in genetic studies and the manifestation of genetic contribution to endothelial function, the mouse genetic linkage/association study for endothelial function has not been formally considered.

Statistical concerns have been raised about traditional mouse linkage and initial GWAS, including low detection power and inflated false positive associations due to population structure, genetic relatedness and limited mapping resolution (81, 141, 211). However, recent advancements in genomic sequence capabilities and mapping algorithms provide denser single nucleotide polymorphisms and reduced false positive associations which minimize the statistical concerns raised with earlier studies (92, 141, 177). In particular, GWAS using a large number of inbred mouse strains has advantages for QTL identification, including a wider range of phenotypic variation and higher reproducibility, detection power and mapping resolution, compared to a traditional cross between two parental strains (81). Utilizing a recently developed mapping algorithm, called the efficient mixed model algorithm (EMMA), can also further correct statistical concerns and optimize computational speed and reliability of the results (141).

Therefore, given the limited evidence regarding the genetic regulation of endothelial function and the improvement in *in silico* mapping methods, we aimed to characterize the genetic contribution to intrinsic endothelial function and to identify quantitative trait loci (QTL)/candidate genes residing in the QTL responsible for

intrinsic endothelial function in mice. Specifically, we examined *in vitro* vasoreactivity in isolated thoracic aortas from 27 different inbred mouse strains, and then conducted GWAS for strain differences in vasoreactivity using EMMA. We hypothesized that intrinsic endothelium-dependent vasorelaxation is largely variable across inbred mouse strains, and the variation is influenced by one or more QTL.

2.2. Methods

2.2.1. Animals

Male mice (n=6-10/strain) from 27 inbred strains were purchased from Jackson Laboratories (Bar Harbor, ME) (Table 2.1). These strains were chosen based on phylogenetically distinct background (214), available sequence data in the efficient mixed model algorithm correction server (141) and the recommendations of the Mouse Phenome Database (101) to cover as much genetic diversity as possible. The list of inbred mouse strains in this study was mostly common (up to 93%) with previous strain-screening studies for cardiovascular phenotypes (18, 50, 250). Upon arrival, mice were familiarized with a new environment at least for one week under a 12h light:dark cycle (7:00AM - 7:00PM) in a controlled temperature (21.0 - 22.0℃). Mice were allowed ad libitum access to food and water during the time. All procedures adhered to the established National Institutes of Health guidelines for the care and use of laboratory animals and were approved by the Institutional Animal Care and Use Committee (IACUC) at Texas A&M University.

Table 2.1. List of inbred mouse strains grouped by phylogenetical background

Group 1	Group 2	Group 3	Group 4	Group 5	Group 6	Group 7
A/J	FVB/NJ	KK/HlJ	C57BL/6J	129S1/SvImJ	DBA/2J	PWD/PhJ
AKR/J	MA/MyJ	NON/LtJ	C57BR/cdJ	129X1/SvJ	I/LnJ	
BALB/cByJ	NOD/LtJ	NZW/LacJ	C58/J	LP/J	SM/J	
C3H/HeJ	SJL/J	NZO/HlLtJ				
CBA/J	SWR/J					
CE/J						
LG/J						
PL/J						

Total 27 inbred mouse strains were separated into seven groups according to their genetic relatedness proposed by Petkov et al. (214). Group 1, Bagg albino derivatives; Group 2, Swiss mice; Group 3, Japanese and New Zealand's inbred strains; Group 4, C57/58 strains; Group 5, Castle's mice; Group 6, C.C. Little's DBA and related strains; Group 7, wild-derived strains**.**

2.2.2. Aortic ring experiments

At 13 weeks of age, mice from 27 inbred strains were weighed and anesthetized by intraperitoneal injection of the cocktail of Ketamine (80 mg/kg) and Xylazine (5 mg/kg). Thoracic aortas were dissected and connective tissue was carefully removed in ice-cold physiological saline solution pH 7.4 (in mmol/l: 118.3 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.2 $MgSO_4$, 1.2 KH_2PO_4 , 25 $NaHCO_3$ and 5.5 glucose) under a microscope. Aortas were cut into 2 mm ring segments of equal length. Each ring segment was suspended in organ chamber of 610M Multi Chamber Myograph System (Danish Myo Technology, Denmark) filled with 8 ml of oxygenated (95% O_2, 5% CO_2) physiological saline solution and allowed to equilibrate at 37˚C for at least 30 minutes. Aortic rings were stretched to the resting tension (9 to 12 mN) determined by the tension-force assessment in response to 25 mM of potassium chloride (KCl). Then cumulative concentration-response curves to phenylephrine (PE: a selective α_1-adrenergic receptor agonist, 10^{-9} to 10^{-5} M) and KCl (a membrane depolarizing agent, 5 to 100 mM) were generated to assess contractile function of aortic rings, while cumulative concentration-response curves to acetylcholine (ACh, muscarinic receptor agonist) and sodium nitroprusside (SNP, nitric oxide donor) (10^{-9} to 10^{-5} M) were generated to assess endothelium-dependent and -independent vasorelaxation, respectively. Proposed mechanisms of vasoconstriction and vasorelaxation induced by these four different vasoactive agents are illustrated in Fig. 2.1. Cumulative concentration-response curves to ACh and SNP were generated after the ring was pre-constricted to 70% of maximal contraction with PE. Doses were added after the response curve reached a plateau from the previous dose.

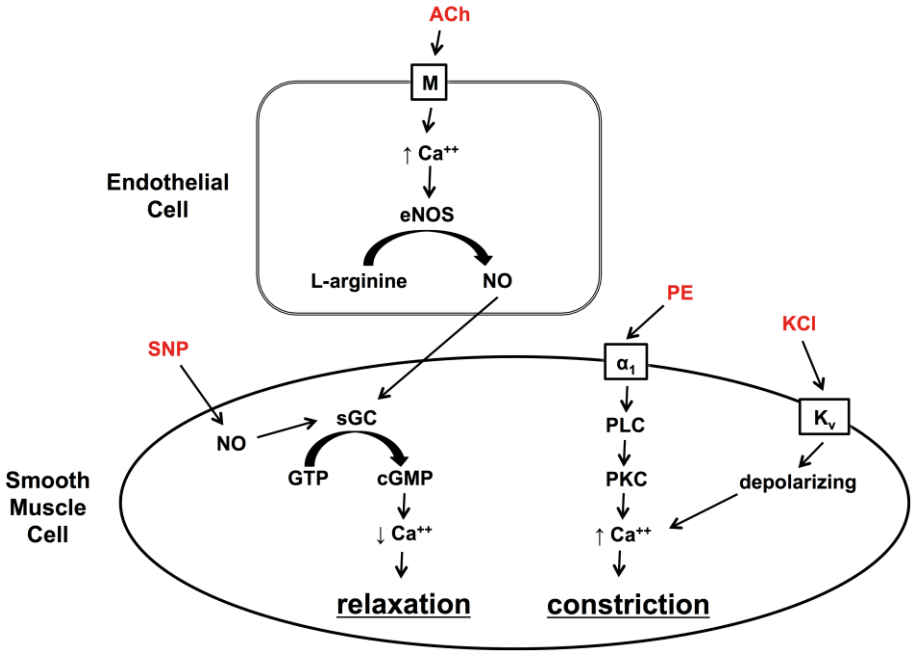

Figure 2.1. Proposed mechanisms of vasorelaxation and vasoconstriction induced by acetylcholine (ACh), sodium nitroprusside (SNP), phenylephrine (PE), and potassium chloride (KCl).

Rigorous experimental standards were applied in order to minimize the impact of non-inherited factors on vasoreactivity. Unused segments of thoracic aorta were snap-frozen in liquid nitrogen and stored at -80. Percent contraction responses were calculated as $[(D_P - D_B)/D_B] \times 100$, where '$D_P$' is the maximal force generated by PE or KCl and 'D_B' is the baseline force. Percent relaxation responses were calculated as $[(D_P - D_D)/(D_P - D_B)] \times 100$, where '$D_P$' is the maximal force pre-generated by PE, 'D_D' is the lowest force generated at a given dose of ACh or SNP and 'D_B' is the baseline force. The half maximal effective and inhibitory concentration (EC_{50} or IC_{50}, respectively) were calculated with absolute values from cumulative concentration-response curves to each vasoactive agent by Prism 6 (GraphPad Software, La Jolla, CA).

2.2.3. Genome-wide association mapping

Genome-wide association mapping (GWAS) for maximal responses and EC_{50} / IC_{50} to four different vasoactive agents were performed with Efficient Mixed Model Algorithm (EMMA) via the web-based server (http://mouse.cs.ucla.edu/emmaserver/). Classical inbred mouse association has been proposed to have potential for spurious (false positive) associations to be generated by unequal relatedness among inbred strains (81, 141, 211). However, EMMA uses a linear mixed-model association with a variance component using a kinship matrix that is based on the genetic relatedness between inbred strains to control for population structure effects, thereby reducing the rate of false positive associations for GWAS (141). EMMA also enables to increase the computational speed and reliability of the results. The association scans were performed

with a 4 million single nucleotide polymorphism panel on the EMMA server. Because wild-derived strains are very dissimilar to classical inbred strains (287) and thus could be a potential source of spurious association (169), PWD/PhJ mice were excluded for EMMA analysis. Each single nucleotide polymorphism was evaluated individually and *p* values were recorded as the strength of the association between phenotype and genotype. Significance threshold *p* value was set using the Bonferroni correction for multiple comparisons (44). For vasoreactivity phenotypes that EMMA results did not contain any *p* values less than Bonferroni correction threshold *p* value, a nominal *p* value of 1.00×10^{-5} was utilized as a suggestive threshold.

If a QTL contained a peak single nucleotide polymorphism, the QTL interval was defined as a region of ± 200 kilobase (kb) from the peak single nucleotide polymorphism. If the two QTL were within 1 megabase (Mb), they were considered one QTL (Berndt 11, Sean 12). Based on the Hybrid Mouse Diversity Panel (HMDP), the majority of peak single nucleotide polymorphisms were found within 1 Mb of either end of a gene and the linkage disequilibrium blocks ($r^2 > 0.7$) had an average distance of 500 kb (14). All significant and suggestive QTL were mapped to NCBI-build-37 mouse assembly using the UCSC Genome Browser (https://genome.ucsc.edu) to identify gene(s) residing in the QTL (229). Rat Genome Database (http://rgd.mcw.edu/) was queried with all significant/suggestive QTL to identify regions of shared synteny with rats or humans (243) and the NHGRI GWAS catalog (https://www.genome.gov/26525384) was searched with genes residing in the QTL to identify conjunction with human GWAS for cardiovascular traits (275).

2.2.4. Statistical analysis

Maximal vasoreactivity and EC_{50}/IC_{50} are presented as mean ± SE and were compared with One-way ANOVA followed by Tukey's post-hoc test. Based on the results from the One-way ANOVA, we calculated two estimates of broad sense heritability, intra-class correlation (*rI*) and coefficient of genetic determination (g_2) which provide an estimate of the contribution of genotype to phenotype (164). The intra-class correlation is an estimated proportion of the total variation that can be explained by differences between strains. The coefficient of genetic determination accounts for the doubling of the additive genetic variance that occurs with inbreeding. Each estimate was calculated using the following equations: $rI = (MS_B - MS_W)/[MS_B + (n - 1)\times MS_W]$ and $g_2 = (MS_B - MS_W)/[MS_B + (2n - 1)\times MS_W]$, where MS_B and MS_W are the between- and within-mean square, respectively, and *n* is the number of animals per strain. Because the number of animals per strain was not the same, *n* was calculated as $n = [1/(a - 1)]\times(n - \sum ni^2)$, where *a* is the number of strains and *ni* is the number of animals in the *i*th strain.

For phenotypic correlation, all possible pairs between individual vasoreactivity responses and/or body weight (BW) were analyzed by Pearson correlation. For genetic correlation (53), all possible pairs between strain means of vasoreactivity responses and/or BW were analyzed by Pearson correlation. Statistical significance was set at $p < 0.05$. EC_{50} or IC_{50} for ACh, SNP and PE transformed with $-\log_{10}$ and EC_{50} for KCl transformed with $\log_{10}$ were used for GWAS. *p* values from GWAS were transformed with $-\log_{10}$ for graphical visualization. All statistics were performed using SPSS 22 (IBM, Armonk, NY).

2.3. Results

Body weight (BW) in 13-wk old male mice varied significantly across 27 inbred mouse strains (F = 33.36, $p = 0.00$). The strain distribution pattern for BW is shown in Fig. 2.2. There was approximately three-fold difference between PWD/PhJ having the lowest (16.83 ± 0.27 g) and NZO/HILtJ having the highest (48.38 ± 2.58 g) BW.

To characterize the genetic contribution to endothelial function, we conducted cumulative concentration-response curves to ACh in isolated thoracic aortas from 27 different inbred mouse strains. We found significant differences in maximal responses (%) to ACh (ACh Max) (F = 7.67, $p = 0.00$) and ACh IC_{50} ($-\log_{10}$) (F = 4.81, $p = 0.00$) among 27 inbred strains (Table 2.2). The strain distribution patterns for ACh Max and ACh IC_{50} are shown in Fig. 2.3. In a panel of 27 genetically diverse inbred mouse strains, there was a nearly two-fold difference between NON/LtJ mice having the lowest (47.91 ± 2.32) and CE/J mice having the highest (94.26 ± 1.23) ACh Max (%) (Fig. 2.3A). ACh IC_{50} was also variable across inbred strains, showing 18.2-fold difference in ACh concentration (M) between the lowest (NZW/LacJ: 6.67 ± 0.14) and the highest (LP/J: 7.93 ± 0.08) inbred strains ($-\log_{10}$) (Fig. 2.3B). In contrast, maximal responses to SNP were not different among 27 inbred strains. All aortic rings were 100% relaxed at SNP concentrations between 10^{-6} and 3×10^{-6} M (data not shown). Analysis of variance showed a strain difference for SNP IC_{50} ($-\log_{10}$) (F = 10.30, $p = 0.00$) (Fig. 2.4 and Table 2.2). I/LnJ had the lowest (7.49 ± 0.22) and FVB/NJ had the highest (8.99 ± 0.07) SNP IC_{50}. There was 31.6-fold difference in SNP concentration (M) between those two strains.

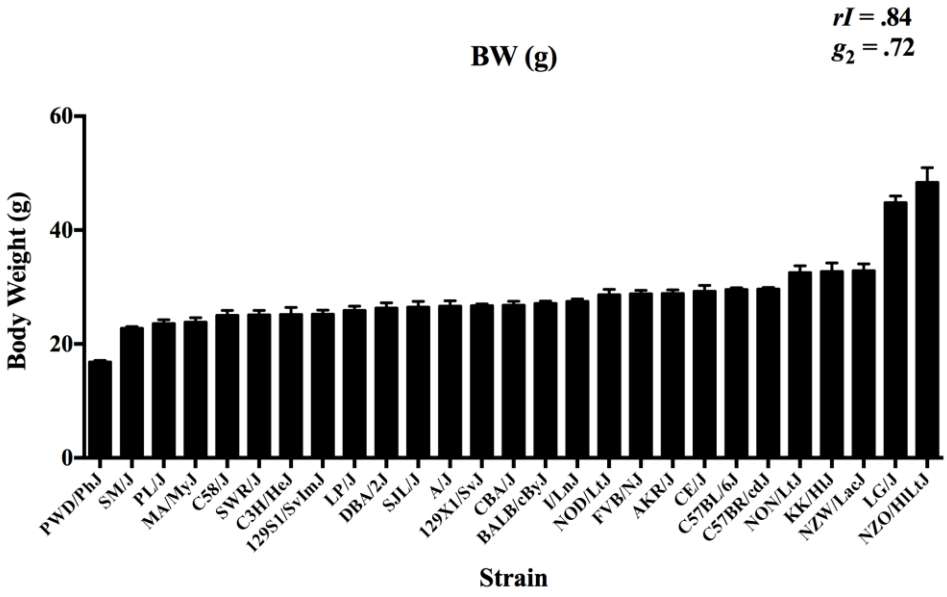

Figure 2.2. Strain distribution pattern for body weight (g) of young male mice from 27 inbred strains. All mice were weighed at 13 weeks of age. *rI*, intra-class correlation; g_2, coefficient of genetic determination. Values are expressed as mean ± SE. *n* = 6-10 mice per strain.

Table 2.2. Statistical differences in vasorelaxation responses to ACh and SNP across 27 inbred mouse strains

Strain	ACh Max (%)	ACh IC_{50} (log_{10})	SNP IC_{50} (log_{10})
129S1/SvImJ	BCD	ABCD	ABCDE
129X1/SvJ	ABCD	ABCD	BCDEF
A/J	BCD	ABCD	ABC
AKR/J	ABC	BCD	HI
BALB/cByJ	CD	ABCD	BCDEF
C3H/HeJ	CD	ABC	ABCD
C57BL/6J	CD	ABCD	BCDEF
C57BR/cdJ	CD	ABCD	BCDEF
C58/J	BCD	CD	DEFGHI
CBA/J	CD	ABC	BCDEF
CE/J	D	ABCD	BCDEF
DBA/2J	ABCD	ABCD	FGHI
FVB/NJ	CD	AB	A
I/LnJ	ABCD	ABCD	I
KK/HlJ	ABCD	ABCD	BCDEFGHI
LG/J	AB	ABCD	GHI
LP/J	CD	A	BCDEFG
MA/MyJ	CD	ABC	BCDEFGH
NOD/LtJ	ABCD	ABCD	CDEFGHI
NON/LtJ	A	ABCD	DEFGHI
NZO/HlLtJ	A	ABCD	EFGHI
NZW/LacJ	AB	D	BCDEFGH
PL/J	CD	AB	DEFGHI
PWD/PhJ	CD	AB	AB
SJL/J	ABCD	ABCD	DEFGHI
SM/J	A	ABCD	GHI
SWR/J	CD	ABCD	BCDEF

Statistical difference was determined by an One-way ANOVA followed by Tukey's post hoc test. Strains not connected by the same letter were significantly different ($p < 0.05$). ACh, acetylcholine; ACh Max, maximal response (%) to ACh; SNP, sodium nitroprusside; IC_{50}, half maximal inhibitory concentration. All aortic rings were 100 % relaxed at SNP concentrations between 10^{-6} and 3×10^{-6} M, thus SNP max (%) were excluded.

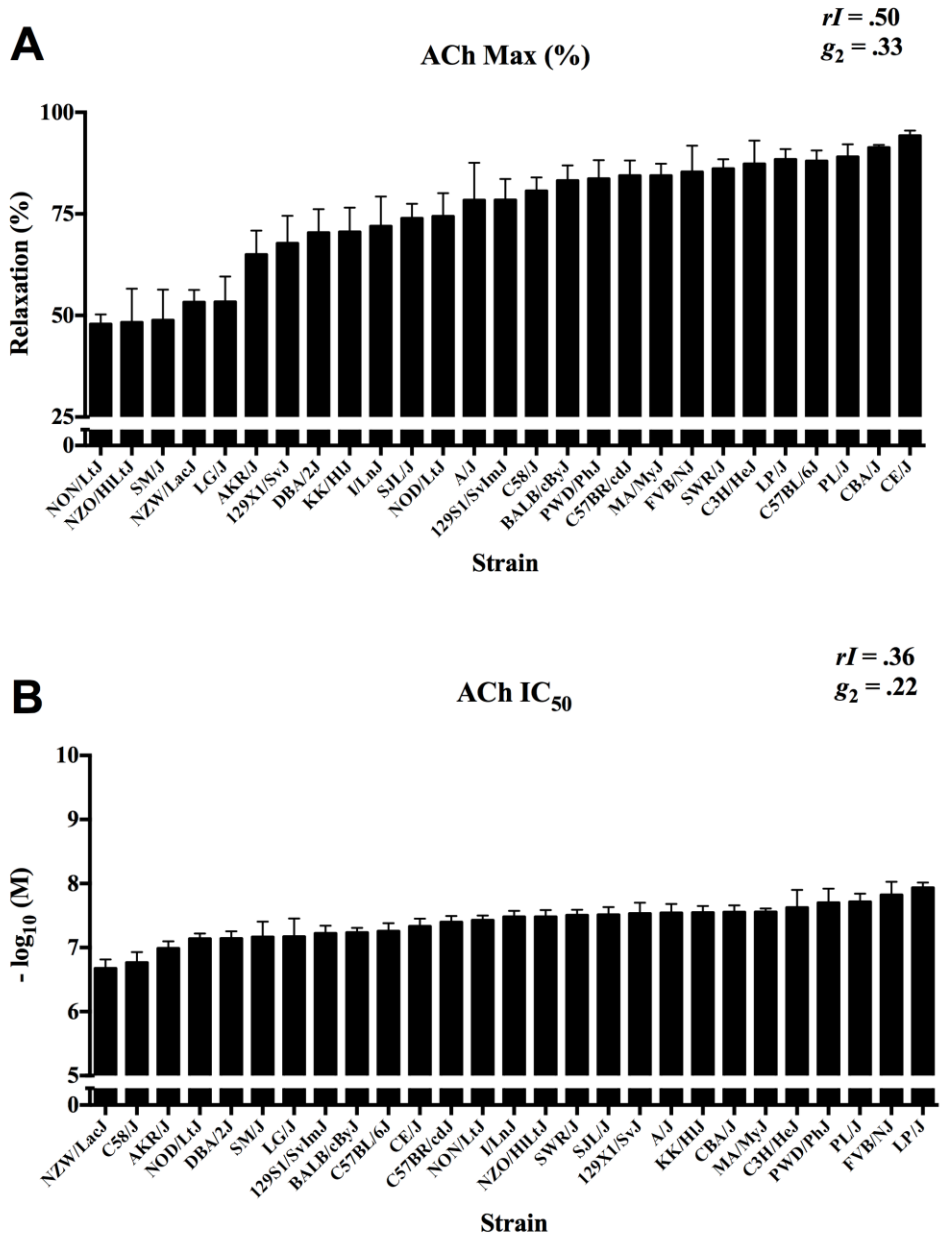

Fig. 2.3. Strain distribution pattern for (A) maximal relaxation responses (%) to acetylcholine (ACh Max) and (B) the half maximal inhibitory concentration in responses to ACh (ACh IC_{50}) in young male mice from 27 inbred strains. *rI*, intra-class correlation; g_2, coefficient of genetic determination. Values are expressed as mean ± SE. *n* = 6-10 mice per strain.

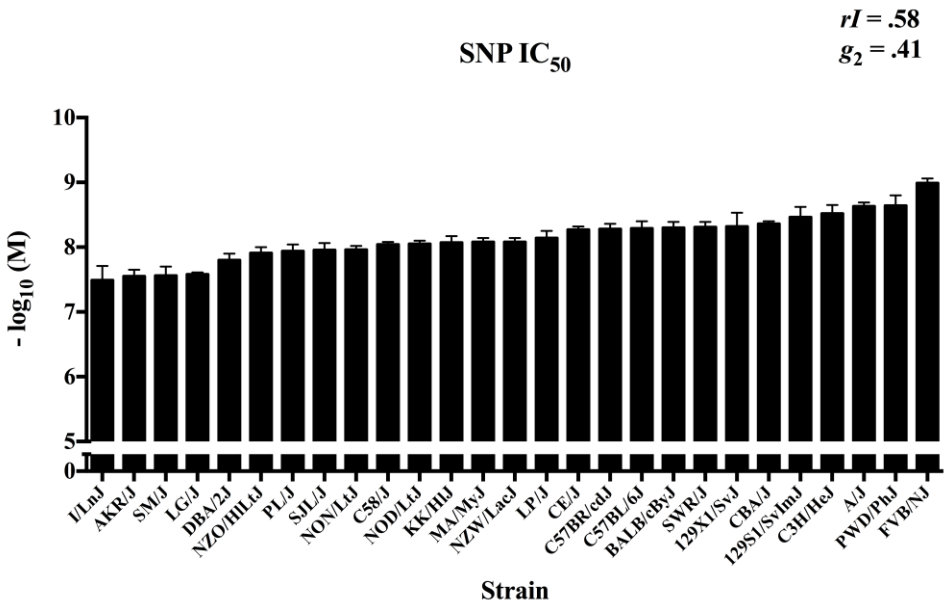

Fig. 2.4. Strain distribution pattern for the half maximal inhibitory concentration in responses to sodium nitroprusside (SNP IC_{50}) in young male mice from 27 inbred strains. *rI*, intra-class correlation; g_2, coefficient of genetic determination. Values are expressed as mean ± SE. *n* = 6-10 mice per strain. All aortic rings were 100 % relaxed at SNP concentrations between 10^{-6} and 3×10^{-6} M, thus maximal relaxation responses (%) to SNP were not shown.

Strain differences in contractile responses to PE and KCL among 27 inbred mice were also observed (Table 2.3). Analysis of variance showed significant differences for PE Max (%) ($F = 12.57$, $p = 0.00$), PE EC_{50} ($-\log_{10}$) ($F = 5.29$, $p = 0.00$), KCl Max (%) ($F = 8.04$, $p = 0.00$), and KCl EC_{50} ($\log_{10}$) ($F = 6.10$, $p = 0.00$). Figs. 2.5 and 2.6 illustrate the strain distribution patterns for vasocontractile responses to PE and KCl, respectively. SM/J had both the lowest PE Max (31.46 ± 0.83 %) and the lowest KCl Max (83.92 ± 4.83 %). In contrast, C58BR/cdJ had the highest PE Max (146.51 ± 2.02 %) and 129S1/SvImJ had the highest KCl Max (183. 21 ± 5.55 %) (Figs. 2.5A and 2.6A). There was 4.7- and 2.2-fold difference between the lowest and the highest strain for PE Max and KCl Max, respectively. Whereas, there was 5.5 -fold difference in PE concentration (M) between the lowest (NZO/HiLtJ: 6.28 ± 0.05) and the highest strain (BALB/cByJ: 7.02 ± 0.07) for PE EC_{50} ($-\log_{10}$) (Fig. 2.5B) and 1.3-fold difference in KCl concentration (M) between the lowest (AKR/J: 1.60 ± 0.00) and the highest strain (FVB/NJ: 1.71 ± 0.04) for KCl EC_{50} ($-\log_{10}$) (Fig. 2.6B).

Phenotypic and genetic correlation analyses for all possible pairs of vasoreactivity responses and/or BW were performed and are indicated in Tables 2.4 and 2.5, respectively. For phenotypic correlations, all vasorelaxion responses were positively correlated each other, while vasocontractile responses were correlated each other with two exceptions: PE EC_{50} *vs.* KCl Max and KCl Max *vs.* KCl EC_{50}. Between vasorelaxion and vasocontractile phenotypes, responses to ACh were not correlated with contractile responses. Only a negative correlation between ACh Max and PE EC_{50} was observed ($r = -0.214$). By comparison, SNP IC_{50} was correlated with contractile

Table 2.3. Statistical differences in vasoconstriction responses to PE and KCl across 27 inbred mouse strains

Strain	PE Max (%)	PE EC_{50} (log_{10})	KCl Max (%)	KCl EC_{50} (log_{10})
129S1/SvImJ	HI	AB	H	AB
129X1/SvJ	HI	AB	FGH	ABCDE
A/J	FGHI	AB	BCDEFG	BCDEF
AKR/J	ABCDE	AB	ABCDEF	F
BALB/cByJ	I	A	EFGH	ABCD
C3H/HeJ	EFGHI	B	BCDEFG	ABCDE
C57BL/6J	BCDEFGHI	AB	BCDEFG	AB
C57BR/cdJ	HI	B	CDEFGH	ABC
C58/J	ABCDEFGHI	AB	BCDEFGH	ABCDE
CBA/J	BCDEFGHI	AB	BCDEFG	ABCDE
CE/J	GHI	AB	BCDEFG	ABCDE
DBA/2J	ABCDEFG	AB	ABCDE	BCDEF
FVB/NJ	EFGHI	AB	BCDEFG	A
I/LnJ	ABCD	AB	ABCD	BCDEF
KK/HlJ	CDEFGHI	AB	ABCD	ABC
LG/J	HI	AB	GH	EF
LP/J	GHI	AB	ABCDE	A
MA/MyJ	A	AB	ABC	BCDEF
NOD/LtJ	FGHI	AB	BCDEFGH	ABCDE
NON/LtJ	DEFGHI	AB	AB	A
NZO/HlLtJ	ABC	B	ABCD	BCDEF
NZW/LacJ	GHI	AB	ABCDEF	BCDEF
PL/J	BCDEFGHI	AB	AB	BCDEF
PWD/PhJ	AB	AB	ABCDEF	BCDEF
SJL/J	ABCDEF	AB	AB	DEF
SM/J	A	B	A	BCDEF
SWR/J	BCDEFGHI	AB	DEFGH	CDEF

Statistical difference was determined by an One-way ANOVA followed by Tukey's post hoc test. Strains not connected by the same letter were significantly different ($p < 0.05$). PE, phenylephrine; KCl, potassium chloride; PE Max, maximal response (%) to PE; KCl Max, maximal response (%) to KCl; EC_{50}, half maximal effective concentration.

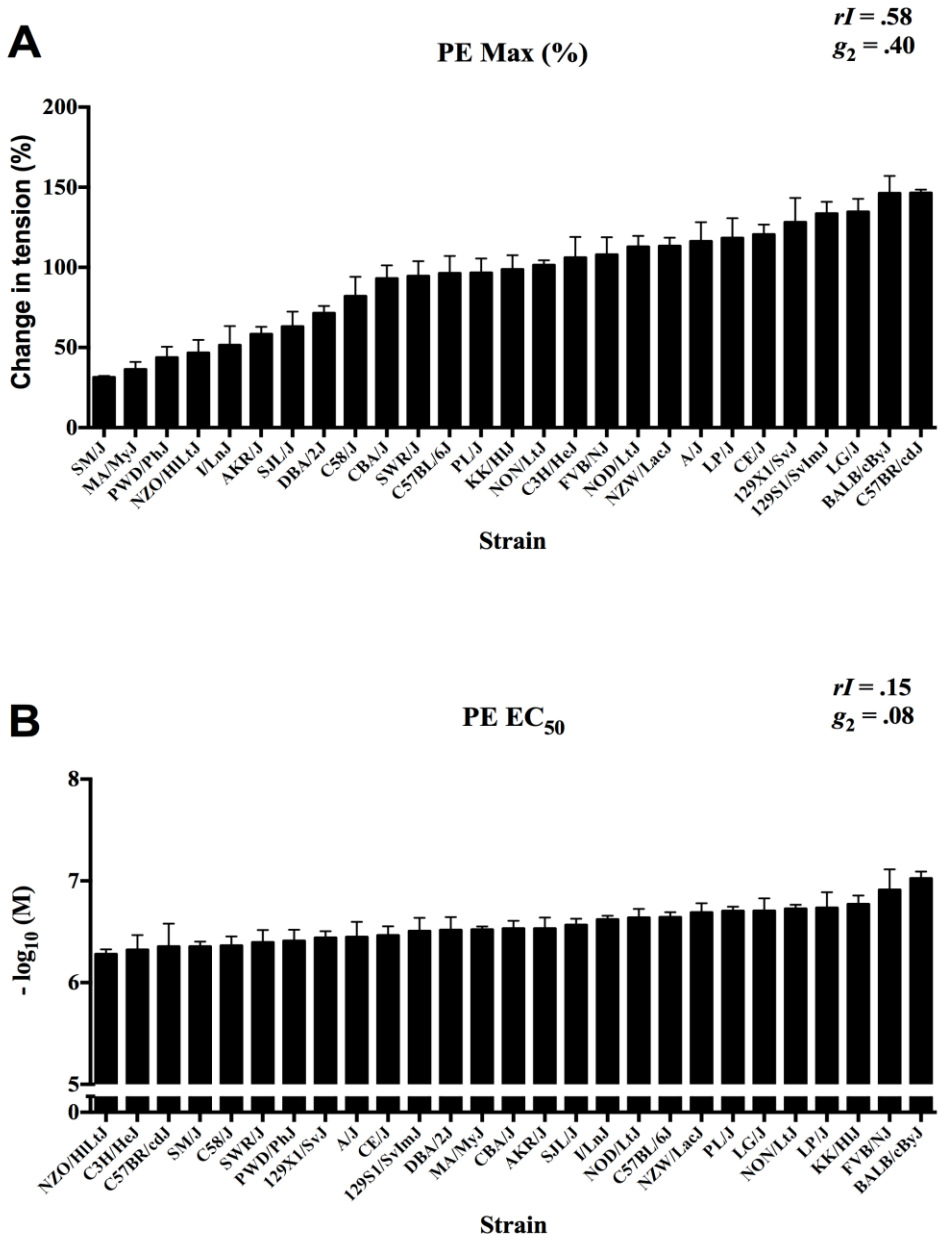

Fig. 2.5. Strain distribution pattern for (A) maximal contractile responses (%) to phenylephrine (PE Max) and (B) the half maximal effective concentration in responses to PE (PE EC_{50}) in young male mice from 27 inbred strains. *rI*, intra-class correlation; g_2, coefficient of genetic determination. Values are expressed as mean ± SE. *n* = 6-10 mice per strain.

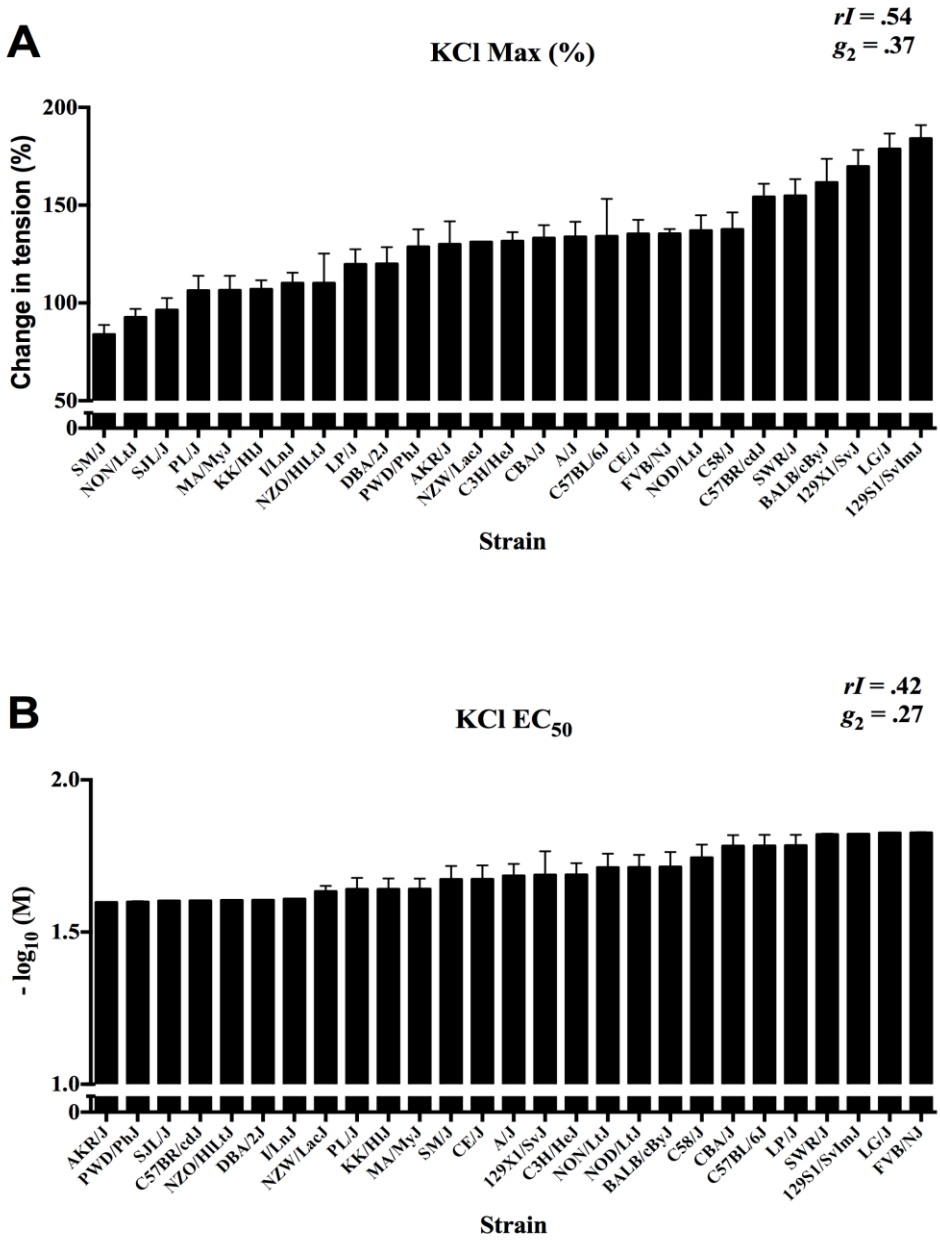

Fig. 2.6. Strain distribution pattern for (A) maximal contractile responses (%) to potassium chloride (KCl Max) and (B) the half maximal effective concentration in responses to KCl (KCl EC_{50}) in young male mice from 27 inbred strains. *rI*, intra-class correlation; g_2, coefficient of genetic determination. Values are expressed as mean ± SE. *n* = 6-10 mice per strain.

Table 2.4. Phenotypic correlations between vasoreactivity responses and/or body weight (BW)

Vasoreactivity		ACh Max (%)	ACh IC_{50} (-log10)	SNP IC_{50} (-log10)	PE Max (%)	PE EC_{50} (-log10)	KCl Max (%)	KCl EC_{50} (-log10)
ACh Max (%)	*r*		**.386***	**.433***	.039	**-.214***	.144	.134
	p		.000	.000	.596	.004	.067	.087
ACh IC_{50} (-log10)	*r*			**.342***	-.144	-.075	-.106	.032
	p			.000	.051	.316	.177	.681
SNP IC_{50} (-log10)	*r*				**.313***	.002	**.210***	**.322***
	p				.000	.984	.009	.000
PE Max (%)	*r*					**.440***	**.473***	**.403***
	p					.000	.000	.000
PE EC_{50} (-log10)	*r*						-.016	**.233***
	p						.840	.003
KCl Max (%)	*r*							.065
	p							.409
BW (g)	*r*	**-.385***	-.147	**-.231***	**.201***	**.181***	.072	.011
	p	.000	.059	.003	.009	.019	.362	.887

Bivariate pearson correlations were conducted for all possible pairs between individual vasoreactivity responses and/or BW. PE, phenylephrine; KCl, potassium chloride; ACh, acetylcholine; SNP, sodium nitroprusside; Max, Maximal response (%); EC_{50}, half maximal effective concentration; IC_{50}, half maximal inhibitory concentration; all aortic rings were 100 % relaxed at SNP concentrations between 10^{-6} and 3×10^{-6} M, thus SNP max (%) were excluded. Statistically significant correlations are bold. *, $p < 0.05$.

Table 2.5. Genetic correlations between vasoreactivity responses and/or body weight (BW)

Vasoreactivity		ACh Max (%)	ACh IC_{50} (-log10)	SNP IC_{50} (-log10)	PE Max (%)	PE EC_{50} (-log10)	KCl Max (%)	KCl EC_{50} (-log10)
ACh Max (%)	*r*		**.403***	**.586***	.235	.034	.234	.227
	p		.037	.001	.237	.868	.239	.254
ACh IC_{50} (-log10)	*r*			**.414***	-.029	.094	-.199	.069
	p			.032	.886	.640	.320	.732
SNP IC_{50} (-log10)	*r*				**.401***	.041	.360	.356
	p				.038	.838	.065	.069
PE Max (%)	*r*					.364	**.699***	**.474***
	p					.062	.000	.012
PE EC_{50} (-log10)	*r*						.031	.287
	p						.877	.146
KCl Max (%)	*r*							**.520***
	p							.005
BW (g)	*r*	**-.568***	-.193	-.315	.170	.097	.104	.058
	p	.002	.335	.110	.395	.631	.607	.775

Bivariate pearson correlations were conducted for all possible pairs between strain means of vasoreactivity responses and/or BW. PE, phenylephrine; KCl, potassium chloride; ACh, acetylcholine; SNP, sodium nitroprusside; Max, Maximal response (%); EC_{50}, half maximal effective concentration; IC_{50}, half maximal inhibitory concentration; all aortic rings were 100 % relaxed at SNP concentrations between 10^{-6} and 3×10^{-6} M, thus SNP max (%) were excluded. Statistically significant correlations are bold. *, $p < 0.05$.

responses except of PE EC_{50}. BW was negatively correlated with vasorelaxation responses with an exception for ACh IC_{50}. On the contrary, BW was positively correlated with contractile responses to PE, but not to KCl. For genetic correlations, ACh and SNP variables also showed significantly positive correlations. Whereas, contractile responses were correlated each other with three exceptions: PE Max *vs.* PE EC_{50}, PE EC_{50} *vs.* KCl Max and KCl Max *vs.* KCl EC_{50}. There were not significant correlations between ACh variables and either PE or KCl variables. Notably, only a negative genetic correlation was identified between BW and ACh Max.

To identify single nucleotide polymorphisms associated with vasoreactivity responses, GWAS for maximal responses and EC_{50} / IC_{50} were performed using EMMA with a 4 million single nucleotide polymorphism panel. Single nucleotide polymorphisms having < 5% of minor allele frequency were automatically excluded from the results by the EMMA server, hence the final results contained approximately 1.27 million single nucleotide polymorphisms. Bonferroni correction for multiple comparisons (44) was used to determine a significant threshold of $p = 3.95 \times 10^{-8}$. Using this threshold, significant associations were only identified for ACh Max and KCl EC_{50}; therefore, a nominal p value of 1.00×10^{-5} was used a suggestive threshold.

Fig. 2.7, Tables 2.6 and 2.7 show GWAS results for endothelium-dependent vasorelaxation. Four significant QTL were identified for ACh Max on 3 different chromosomes; Chrs. 1 (145.37-145.77 and 148.45-148.85 Mb), 2 (149.58-149.98 Mb) and 19 (22.20-22.79 Mb). At the suggestive level, 18 QTL were found for ACh Max on 12 different chromosomes. In contrast, no significant QTL was identified for ACh IC_{50}.

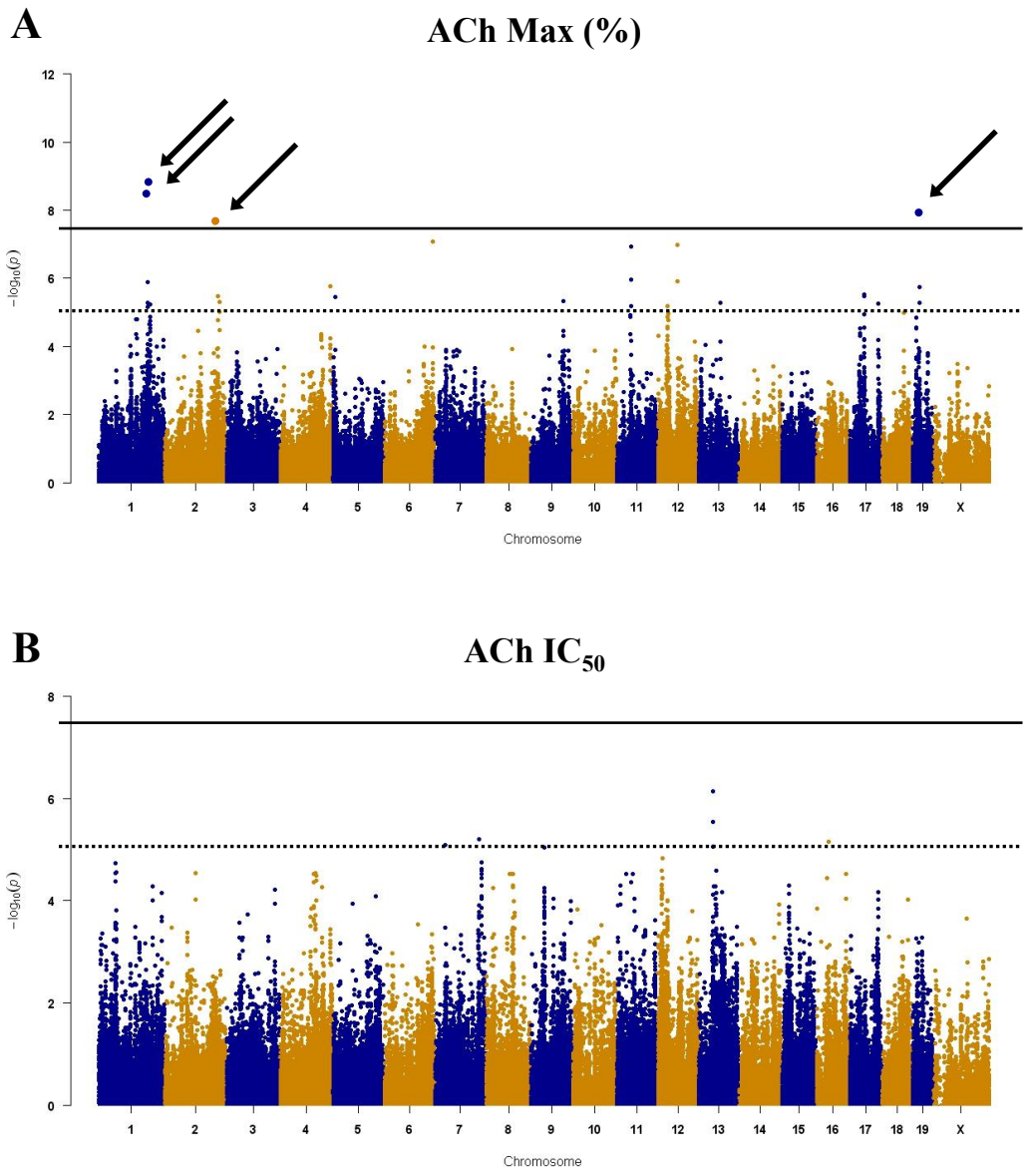

Figure 2.7. Genome-wide association mapping (GWAS) for (A) maximal relaxation responses (%) to acetylcholine (ACh Max) and (B) the half maximal inhibitory concentration in response to ACh (ACh IC_{50}) in young male mice from 26 inbred strains. GWAS was conducted using efficient mixed model algorithm with 4 million single nucleotide polymorphisms. The *x*-axis indicates chromosomes and *y*-axis indicates *p*-values transformed by $-\log_{10}$. The solid horizontal line indicates Bonferroni-corrected significant cut-off threshold (*p* value $<3.95\times 10^{-8}$), while the dashed line indicates suggestive cut-off threshold (*p* value $<1.00\times 10^{-5}$).

Table 2.6. Significant and suggestive QTL found by GWAS for vasorelaxation responses to ACh

Trait	Significance	Chr.	QTL Interval (Mb)	Peak *marker*	Location (Mb)	*p* value	Allele
ACh Max (%)	**Significant**	1	145.37-145.77	rs30978316	145.57	3.41e-09	A/G
		1	148.17-148.96	rs31892646	148.66	1.43e-09	A/T
		2	149.58-149.98	rs6343262	149.78	2.28e-08	G/T
		19	22.20-22.79	rs37653496	22.40	1.21e-08	C/T
	Suggestive	1	154.89-155.29	rs32589931	155.09	5.89e-06	A/G
		2	157.82-158.22	rs27320451	158.02	3.40e-06	C/T
		2	163.18-163.58	rs28281229	163.38	4.98e-06	A/G
		4	150.58-150.98	rs32323516	150.78	1.74e-06	C/T
		5	9.55-10.08	rs37664807	9.87	3.56e-06	G/T
		6	146.07-146.47	rs38688580	146.27	8.59e-08	C/T
		9	98.19-98.76	rs33165797	98.46	4.72e-06	C/T
		11	44.34-45.24	rs26915649	44.68	1.17e-07	G/T
		12	30.49-30.89	rs29151171	30.69	6.53e-06	A/G
		12	60.81-61.21	rs45948495	61.01	1.03e-07	C/G
		13	66.77-67.17	rs48005777	66.97	5.29e-06	C/T
		17	45.29-45.92	rs45985354	45.51	2.92e-06	A/G
		17	86.74-87.14	rs33082540	86.94	5.55e-06	G/T
		18	66.74-67.15	rs36401271	66.95	9.97e-06	A/T
ACh IC_{50}	Suggestive	7	32.00-32.40	rs50008818	32.20	7.99e-06	C/T
		7	132.38-132.80	rs32985074	132.58	6.30e-06	C/T
		9	41.80-42.20	rs30322841	42.00	8.95e-06	C/T
		13	43.15-43.84	rs29735389	43.35	7.11e-07	A/C
		16	38.39-38.79	rs51898661	38.59	7.04e-06	G/T

Quantitative trait loci (QTL) having *p* values $\leq 3.95\times10^{-8}$and 1.00×10^{-5} were considered significant and suggestive, respectively. The QTL intervals were estimated to be 400 kb centered around the single peak *SNP*. If two QTL were separated < 1Mb, they were considered one QTL. The reference single nucleotide polymorphism (rs) numbers for peak markers were identified using the UCSC Genome Browser Chr., chromosome; ACh, acetylcholine; ACh Max, maximal response (%) to ACh; IC_{50}, half maximal inhibitory concentration.

Table 2.7. Protein-coding genes residing in significant and suggestive QTL associated with vasorelaxation responses to ACh

Trait	Chr.	Significance	QTL interval (Mb)	Protein coding genes
ACh Max (%)	1	**Significant**	145.37-145.77	B3galt2, **Glrx2**, Uchl5
	1	**Significant**	148.17-148.96	**Fam5c**
	1	Suggestive	154.89-155.29	NMnat2, Lamc1, Lamc2
	2	**Significant**	149.58-149.98	Syndig1, Zfp120, Tmem90b
	2	Suggestive	157.82-158.22	Rprd1b, Tgm2, Tti1, Bpi, Lbp, Snhg11
	2	Suggestive	163.18-163.58	Fitm2, Gdap1l1, Hnf4a, Jph2, Ttpa1, Pkig, Serinc3, Ada
	4	Suggestive	150.58-150.98	Camta1
	5	Suggestive	9.55-10.08	Grm3
	6	Suggestive	146.07-146.47	Itpr2
	9	Suggestive	98.19-98.76	Copb2, Mrps22, Nmnat3, Rbp1, Rbp2, Prr23a
	11	Suggestive	44.34-45.24	Ebf1, Rnf145
	12	Suggestive	30.49-30.89	Myt1l, Pxdn, Tpo, Sntg2
	12	Suggestive	60.81-61.21	
	13	Suggestive	66.77-67.17	Zfp640, Uqcrb, Mterfd1, Ptdss1, Zfb712
	17	Suggestive	45.29-45.92	Cdc5l. Spats1, Aars2, Tcte1, Nfkbie, Slc35b2, Tmem151b, Hsp90ab1, Ent1, Slc29a1, Capn11, Mrpl14
	17	Suggestive	86.74-87.14	Prkce
	18	Suggestive	66.74-67.15	MC4r
	19	**Significant**	22.20-22.79	**Trpm3**
ACh IC_{50}	7	Suggestive	32.00-32.40	Apbh, Abpe
	7	Suggestive	132.38-132.80	Jmjd5, Nsmce1, Il4ra, Il21r, Gtf3c1
	9	Suggestive	41.80-42.20	Sorl1, Sc5d, Tecta
	13	Suggestive	43.15-43.84	Phactr1, Tbc1d7, Gfod1, Sirt5, Nol7, Ranbp9, Ccdc90a, Rnf182
	16	Suggestive	38.39-38.79	Pla1a, Cd80, Adprh, Poglut1, Tmem39a, Cdgap, B4galt4, Upk1b

Quantitative trait loci (QTL) having p values $\leq 3.95\times10^{-8}$and 1.00×10^{-5} were queried into the UCSC Genome Browser using NCBI-build-37 mouse assembly to search genes residing in the QTL intervals. ACh, acetylcholine; ACh Max, maximal response (%) to ACh; IC_{50}, half maximal inhibitory concentration; Chr., chromosome; QTL, quantitative trait loci. Proposed candidate genes are bold and putative candidate genes are underlined.

Five suggestive QTL were found for ACh IC_{50}. These QTL were identified on 4 different chromosomes; Chrs. 7 (32.00-32.40 Mb and 132.38-132.80 Mb), 9 (41.80-42.20 Mb), 13 (43.15-43.84 Mb), and 16 (38.39-38.79 Mb).

GWAS also revealed significant and suggestive QTL for responses to SNP, PE and KCl (Figs. 2.8, 2.9 and 2.10 and Table 2.8). For KCl EC_{50}, one significant QTL was found on X chromosome (101.61-102.40 Mb). In contrast, no significant QTL was detected for other vasoreactivity responses. Several QTL were identified at the suggestive level; 15 suggestive QTL on 10 different chromosomes for SNP IC_{50}, 9 suggestive QTL on 8 different chromosomes for PE Max, 7 suggestive QTL on 7 different chromosomes for PE EC_{50}, 11 suggestive QTL on 8 different chromosomes for KCl Max, and 8 suggestive QTL were identified on 6 different chromosomes.

Three suggestive QTL for ACh Max overlapped with QTL for SNP IC_{50} on 3 different chromosomes; Chr. 2 (163.2–163.6 Mb), 11 (44.3–45.2 Mb) and 17 (86.7–87.1 Mb), whereas none of suggestive QTL for ACh IC_{50} overlapped with any other QTL. QTL for contractile responses did not overlap each other. One suggestive QTL (Chr. 2: 163.4–163.6 Mb) was common to three traits, ACh Max, SNP IC_{50} and PE Max.

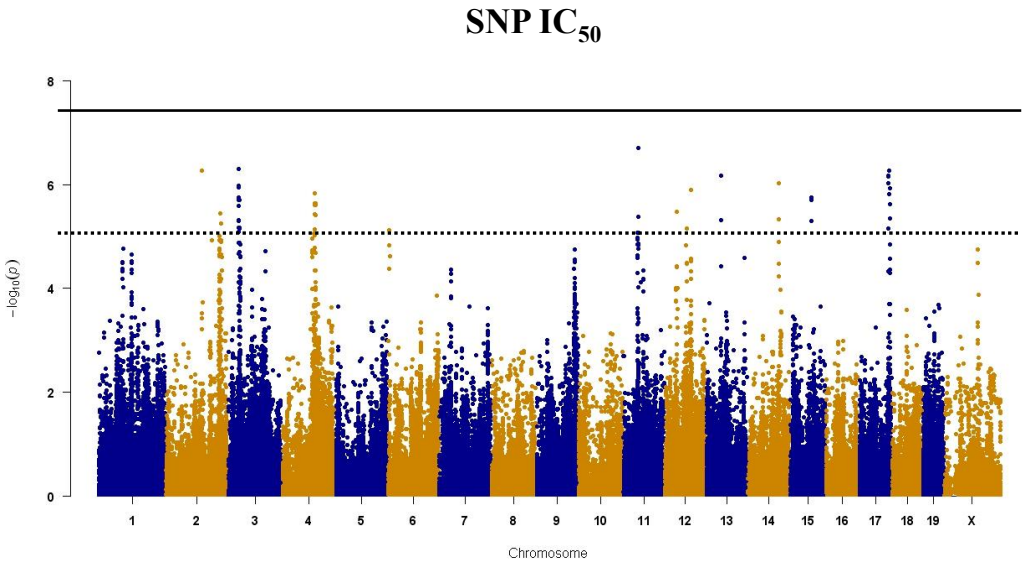

Figure 2.8. Genome-wide association mapping (GWAS) for the half maximal inhibitory concentration in responses to sodium nitroprusside (SNP IC_{50}) in young male mice from 26 inbred strains. GWAS was conducted using efficient mixed model algorithm with 4 million single nucleotide polymorphisms. The *x*-axis indicates chromosomes and *y*-axis indicates *p*-values transformed by $-\log_{10}$. The solid horizontal line indicates Bonferroni-corrected significant cut-off threshold (*p* value $<3.95\times10^{-8}$), while the dashed line indicates suggestive cut-off threshold (*p* value $<1.00\times 10^{-5}$).

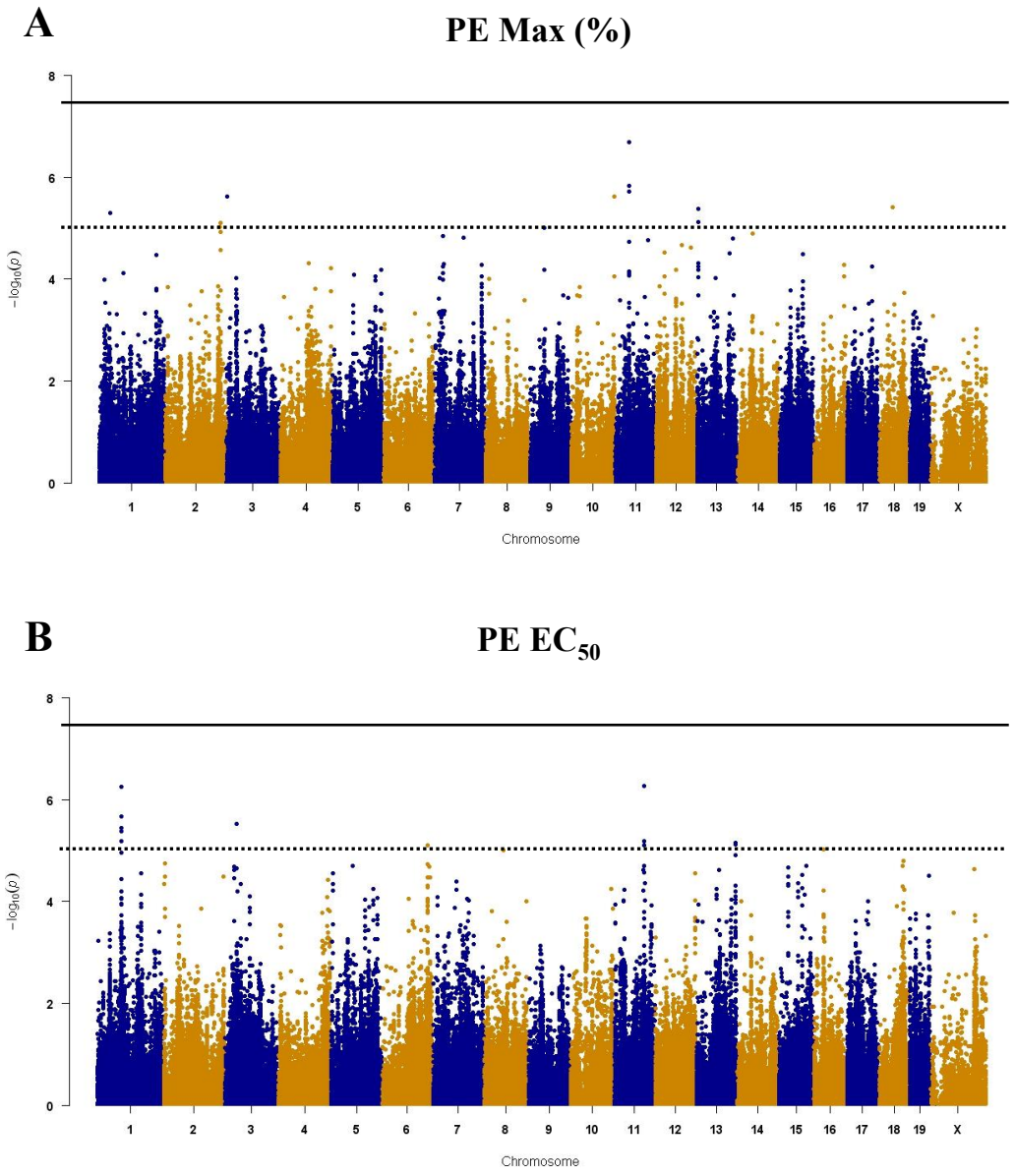

Figure 2.9. Genome-wide association mapping (GWAS) for (A) maximal contractile responses (%) to phenylephrine (PE Max) and (B) the half maximal effective concentration in responses to PE (PE EC_{50}) in in young male mice from 26 inbred strains. GWAS was conducted using efficient mixed model algorithm with 4 million single nucleotide polymorphisms. The *x*-axis indicates chromosomes and *y*-axis indicates *p*-values transformed by $-\log_{10}$. The solid horizontal line indicates Bonferroni-corrected significant cut-off threshold (*p* value $<3.95\times10^{-8}$), while the dashed line indicates suggestive cut-off threshold (*p* value $<1.00\times 10^{-5}$).

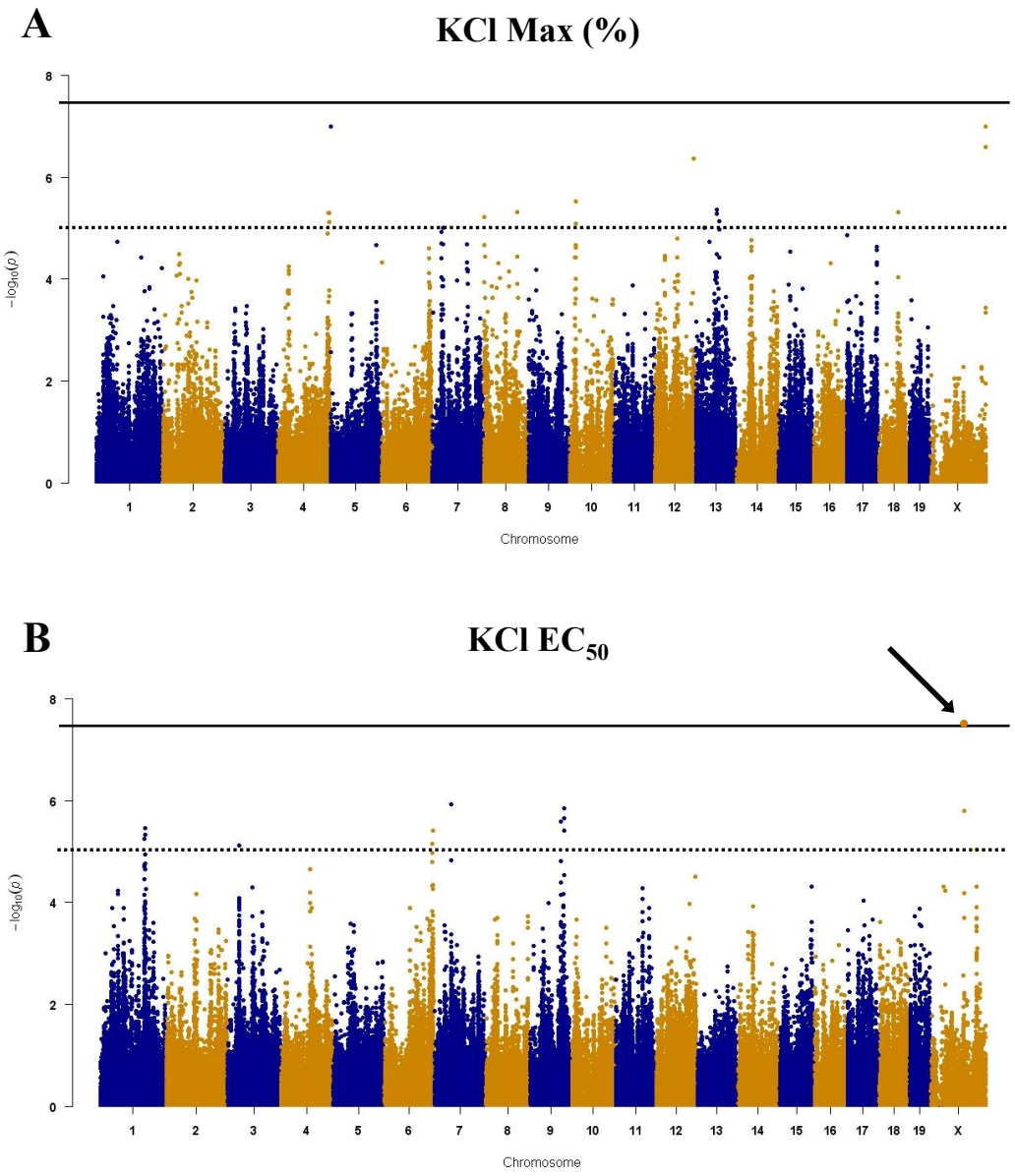

Figure 2.10. Genome-wide association mapping (GWAS) for (A) maximal contractile responses (%) to potassium chloride (KCl Max) and (B) the half maximal effective concentration in responses to KCl (KCl EC_{50}) in in young male mice from 26 inbred strains. GWAS was conducted using efficient mixed model algorithm with 4 million single nucleotide polymorphisms. The *x*-axis indicates chromosomes and *y*-axis indicates *p*-values transformed by $-\log_{10}$. The solid horizontal line indicates Bonferroni-corrected significant cut-off threshold (*p* value < 3.95×10^{-8}), while the dashed line indicates suggestive cut-off threshold (*p* value < 1.00×10^{-5}).

Table 2.8. Significant and suggestive QTL found by GWAS associated with vasoreactivity responses to SNP, PE and KCl

Trait	Significance	Chr.	QTL Interval (Mb)	Peak *marker*	*p* value	overlapping QTL (Mb) with ACh Max
SNP IC_{50}	Suggestive	2	107.51-107.91	rs27490041	5.27e-07	
		2	159.24-160.04	rs28281159	3.53e-06	
		2	163.18-163.58	rs28281229	5.53e-06	163.18-163.58
		3	33.54-35.63	rs29894681	4.91e-07	
		4	94.28-94.68	rs28085124	1.44e-06	
		6	4.28-4.68	rs30170734	7.48e-06	
		11	43.99-45.24	rs26897621	1.95e-07	44.34-45.24
		12	36.02-36.42	rs47949668	3.37e-06	
		12	66.34-66.74	rs29128270	6.87e-06	
		12	78.01-78.56	rs29174383	1.25e-06	
		13	43.99-44.39	rs30144354	6.69e-07	
		14	93.44-94.30	rs31281733	9.34e-07	
		15	63.80-64.12	rs31584477	1.76e-06	
		17	86.74-88.20	rs29766620	5.44e-07	86.74-87.14
		17	90.58-91.36	rs33679213	1.19e-06	
PE Max (%)	Suggestive	1	35.46-35.86	rs32051270	5.03e-06	
		2	163.41-143.81	rs27331097	9.30e-06	163.41-163.58
		2	165.69-166.09	rs27295338	7.85e-06	
		3	3.03-3.43	rs29618455	2.36e-06	
		9	45.77-46.22	rs32676184	9.80e-06	
		10	129.68-130.11	rs46235569	2.36e-06	
		11	42.87-43.56	rs28205221	1.47e-06	
		13	6.21-7.09	rs50398136	4.14e-06	
		18	42.06-42.46	rs63933869	3.83e-06	
PE EC_{50}	Suggestive	1	73.82-74.68	rs36403436	2.14e-06	
		3	37.10-37.50	rs30063078	2.95e-06	
		6	135.78-136.18	rs36997108	7.75e-06	
		8	59.85-60.25	rs31384283	9.89e-06	
		11	89.72-90.15	rs27107288	5.43e-07	
		13	119.05-119.96	rs29834625	7.47e-06	
		16	30.32-30.72	rs4168249	9.36e-06	

Table 2.8 Continued

Trait	Significance	Chr.	QTL Interval (Mb)	Peak *marker*	*p* value	overlapping QTL (Mb) with ACh Max
KCl Max (%)	Suggestive	4	152.98-153.38	rs32999511	5.05e-06	
		5	3.16-3.56	rs31228881	1.01e-07	
		7	33.01-33.41	rs36239097	9.68e-06	
		8	4.52-5.08	rs47036401	6.03e-06	
		8	100.37-100.77	rs33356432	4.79e-06	
		10	18.38-18.82	rs29358047	2.95e-06	
		12	116.63-117.03	rs47495711	4.20e-07	
		13	27.25-27.65	rs30054551	9.68e-06	
		13	63.35-63.84	rs29249644	4.36e-06	
		13	70.63-71.04	rs50665869	7.25e-06	
		18	58.57-58.98	rs49997899	4.86e-06	
KCl EC_{50}	**Significant**	X	101.61-102.40	rs29078805	3.24e-08	
	Suggestive	1	134.91-135.65	rs32757676	5.55e-06	
		1	136.94-137.34	rs37503025	3.41e-06	
		3	36.53-36.93	rs3151465	7.44e-06	
		6	145.30-146.07	rs38919844	3.81e-06	
		7	49.90-50.38	rs37494318	1.19e-06	
		9	92.56-92.96	rs29597520	2.55e-06	
		9	102.08-102.56	rs29840346	1.42e-06	
		X	138.49-139.12	rs29287900	9.16e-06	

Quantitative trait loci (QTL) having *p* values ≤ 3.95e-08 and 1.00e-05 were considered significant and suggestive, respectively. The QTL intervals were estimated to be 400 kb centered around the single peak *marker*. If two QTL were separated < 1Mb, they were considered one QTL. The reference single nucleotide polymorphism (rs) numbers for peak markers were identified using the UCSC Genome Browser. Chr., chromosome; SNP, sodium nitroprusside; PE, phenylephrine; KCl, potassium chloride; PE and KCl Max, maximal response (%) to PE and KCl, respectively; IC_{50}, half maximal inhibitory concentration; EC_{50}, half maximal effective concentration.

2.4. Discussion

Despite accumulating evidence indicating that endothelial function is genetically regulated, the genetic basis for endothelial function still remains to be unclear. Here, we assessed vasoreactivity responses to vasoactive agents in isolated thoracic aortas from 27 strains of genetically diverse inbred mice at 13 weeks of age under controlled environmental conditions. The strain-dependent variation in vasoreactivity observed in the present study enabled us to conduct genome-wide association mapping to identify quantitative trait loci responsible for the variation. The main findings of the present study were: 1) vasoreactivity responses in isolated aortas varied across 27 inbred mouse strains; 2) there were some correlations between vasoreactivity responses; 3) several significant and suggestive QTL were identified for the variation in endothelium-dependent relaxation and prospective candidate genes were found in those QTL. Our findings provide essential genetic information underlying individual susceptibility to endothelial dysfunction, thus insights into identifying potential therapeutic targets to prevent or treat endothelial dysfunction.

Control of vascular function is important for blood pressure regulation and regional distribution of blood flow. Impaired regulation of vascular function, especially endothelial dysfunction, is associated with various forms of cardiovascular disease. Limited data suggests that genetic factors contribute to the variation in vascular function in humans and animals. In the present study, significant differences in endothelium-dependent vasorelaxation were found in aorta from 27 inbred strains of mice (Fig. 2.3 and Table 2.2), with an approximately 2-fold difference between the highest and lowest

responding strains in maximal response to ACh. Our results provide an expanded phenotype dataset (≥ 3-fold) of endothelium-dependent vasorelaxation measurements over previous studies (41, 233). Ryan et al. reported differences in endothelium-dependent vasorelaxation across 7 different inbred mouse strains (male, 16 to 22 weeks old) (233), of which four inbred strains were included in the present study. In general the strain distribution and magnitude of responses to ACh were similar to Ryan et al. with one exception. The authors found that two 129 sub-strains (129P3/J and 129X1/SvJ) had significantly attenuated endothelium-dependent vasorelaxation to ACh (< 20% maximal relaxation) compared with other 5 strains (A/J, Balb/cJ, C3HeB/FeJ, C57BL/6J, and SWR/J). In the present study, 129X1/SvJ had significantly lower ACh Max compared with A/J, SWR/J and C57BL/6J, but the maximum response for this strain was greater than 50%. (Fig. 2.3A). Chen et al. also measured vasorelaxation responses to ACh in SJL, FVB and C3H inbred strains (41). Although the magnitude of responses was somewhat different between studies, the strain distribution pattern in the present study was similar to Chen et al.. In the present study, we also found strain-dependent variation in ACh IC_{50} among 27 inbred mouse strains (Fig. 2.3B and Table 2.2). Chen et al. did not observe differences in ACh IC_{50} across three strains, despite differences in maximal responses. However, in two consomic panels of rats created from the introgression of a single chromosome from one inbred rat strain (Brown Norway) onto other two inbred rat strains (Dahl salt sensitive and Fawn Hooded Hypertensive), Kunert and colleagues found significant differences in ACh IC_{50} between several consomic lines and the parental strain (150, 151), demonstrating that sensitivity to ACh would be regulated by

genetic background. Together, our findings of variation in ACh max and ACh IC_{50} across 27 inbred mouse strains support the notion that endothelial function is influenced by genetic background.

Maximal responses to SNP were not different among inbred mouse strains since all aortic rings were 100% relaxed at SNP concentrations between 10^{-6} and 3×10^{-6} M (data not shown). These results are consistent with previous findings that the ability of vascular smooth muscle to dilate in response to high doses ($> 10^{-7}$ M) of nitric oxide (NO) donors is not variable in young animals (233). In contrast, the IC_{50} for SNP varied across inbred mouse strains (Fig. 2.4 and Table 2.2). In their three-strain comparison, Chen et al. found that SNP IC_{50} was significantly different in SJL compared with FVB and C3H strains (41). Thus, our results further provide evidence that genetic background affects sensitivity of vascular smooth muscle to NO.

The evidence for genetic regulation of vasocontractile function is limited. Posti et al. reported inter-individual variation in contractile responses to PE (217) and Stein et al. found marked ethnic differences in sensitivity to PE (251). Using the consomic rat panels, Kunert and colleagues identified that sensitivity to PE varied among consomic strains (150, 151), suggesting that smooth muscle contractile responses to PE might be regulated by multiple chromosomes. In the present study, responses to PE varied by about five-fold, whereas the responses to KCl varied by two-fold. These ranges are larger than those previously reported for responses to contractile agents in mouse aorta (41, 233), but our study incorporates a much larger number of strains. Although data regarding the genetic regulation of contractile function are limited, our findings of

variation in contractile responses to both PE and KCl among 27 inbred strains (Figs. 2.5 and 2.6) provide evidence that contractile functions are also regulated by genetic background.

ACh-induced endothelium-dependent vasorelaxation was moderately, but significantly, correlated with SNP-induced endothelium-independent relaxation (Table 2.4). ACh is a muscarinic receptor agonist that stimulates the release of vasorelaxing molecules, e.g. NO, prostacyclin and hyperpolarizing factors from endothelial cells. Those relaxing molecules released from endothelial cells diffuse into vascular smooth muscles, eventually causing muscle relaxation (55, 158). Whereas, SNP is a NO donor that diffuses directly into vascular smooth muscle cells, thus inducing muscle endothelium-independent relaxation (142). Our finding of modest phenotypic correlations between responses to ACh and SNP (r = 0.342 to 0.433) would be expected because both agents increase the influx of NO into vascular smooth muscle. Significant genetic correlations between responses to ACh and SNP IC_{50} (Table 2.5) indicate that vasorelaxation responses to ACh and SNP would be influenced, in part, by common genetic factors.

Previous studies have demonstrated that exaggerated contractile responses to vasoconstrictors are associated with attenuated endothelium-derived NO because α-adrenoreceptor agonists, e.g. PE and norepinephrine, stimulate not only α-adrenoreceptor on smooth muscle, but also the release of NO from endothelium (58, 124). In the present study, responses to ACh were not correlated with contractile responses (except for between ACh Max and PE EC_{50}). This finding implies that strain

differences in endothelium-dependent vasorelaxation observed in the present study might not be mainly due to differences in NO pathways. In addition, the absence of genetic correlations between responses to ACh and contractile responses (Table 2.5) demonstrates that genetic regulation would be different between endothelium-dependent vasorelaxation and vasocontractile responses. On the contrary, SNP IC_{50} and contractile responses were partly correlated both phenotypically and genetically, raising the possibility that relaxation responses to NO and contractile responses in smooth muscle are influenced partly by common genetic factors.

BW was inversely correlated with ACh Max and SNP IC_{50}, but positively correlated with contractile responses to PE (Table 2.4). These results would fit in with previous clinical and epidemiological studies demonstrating that BMI or body fatness is inversely associated with endothelial function (1, 254). However, we did not assess body composition and body length in the present study.

Phenotyping a large number of inbred mouse strains with a range of genetic diversity enabled us to perform GWAS to identify QTL responsible for endothelium-dependent vasorelaxation, as well as endothelium-independent relaxation and contractile responses. In the present study, four single nucleotide polymorphisms were significantly associated with ACh Max (Table 2.6) and these are all located in non-coding regions: 1 in intergenic and 3 intronic regions. Pauli et al. currently reported that approximately 46% of genetic variants curated from the NHGRI GWAS catalog (https://www.genome.gov/ 26525384) were enriched within functional element-residing non-coding areas annotated by The Encyclopedia of DNA Elements (ENCODE) (https://www.encodeproject.org/)

(68). These data indicate that genetic variants residing in non-coding regions can function as transcriptional regulators for neighboring genes. Therefore, single nucleotide polymorphisms residing in non-coding regions might have underlying functional significance that regulates investigations.

GWAS have shown potentials to not only provide a chance to identify novel single nucleotide polymorphisms, but also confirm the results of previous candidate gene studies. In the present study, however, well-known endothelial genes, e.g. *eNOS. CYBA* or superoxide dismutase 1 (*SOD-1*), were not identified in any of significant/suggestive QTL associated with the variation in endothelium-dependent vasorelaxation (Table 2.7). This implies that strain differences in endothelium-dependent vasorelaxation observed in the present study might be attributed to previously unsuspected pathways. For example, family with sequence similarity 5, member c (*Fam5c*) gene, also known as bone morphogenetic protein/retinoic acid inducible neural specific 3, is located in a significant QTL on Chr. 1 (148.45-148.85 Mb). The peak single nucleotide polymorphism resides in intron 6 of this gene. The function of the Fam5c gene in vascular function has not been directly characterized, but *Fam5c* mRNA was shown to be upregulated in response to inflammatory stimuli in endothelial cells (234). Expression of *Fam5c* in endothelium of human coronary arteries was associated with expression of vascular adhesion molecules (*ICAM, VCAM*). In a human GWAS study conducted by Connelly et al., the human ortholog of *Fam5c* was strongly associated with myocardial infarction (49). The author confirmed that the peak single nucleotide polymorphism for this gene showed allele-specific expression in human aorta. Thus, *Fam5c* is a reasonable candidate gene to

be investigated further for its role in endothelial function regulation, particularly, in inflammatory response process.

A significant QTL for ACh Max on Chr. 19 (22.20-22.60 Mb) contains only one gene (Table 2.7), transient receptor potential cation channel, subfamily M, Member 3 (*Trpm3*) and the peak single nucleotide polymorphism is located in intron 1. *Trpm3* belongs to the family of transient receptor potential channels which are currently considered as proteins mediating diverse non-voltage-gated calcium entry pathways in vascular and communicating endothelial cells (100, 292). Its activity is increased by calcium store depletion and muscarinic receptor activation (100). Accordingly, we would also consider *Trpm3* as one of candidate genes regulating endothelial function. Physiological and molecular analyses, e.g. gene/protein expression and gene-targeting studies, are required to identify and prove the role of proposed candidate genes in endothelial function regulation.

The other two significant QTL for ACh Max (%) contain genes that have not been formerly characterized for their role in vascular function or their contribution to cardiovascular disease. A significant QTL on Chr. 1 (145.37-145.77 Mb) contains 3 protein coding genes; UDP-Gal:BetaGlcNAc Beta 1,3-Galactosyltransferase, Polypeptide 2 (*B3galt2*), Glutaredoxin 2 (*Glrx2*) and Ubiquitin Carboxyl-Terminal Hydrolase (*Uchl5*) (Table 2.7). The peak single nucleotide polymorphism was located in an intergenic region. *Glrx2* is known to play a role in the maintenance of mitochondrial redox homeostasis via the involvement in response to hydrogen peroxide and regulation of apoptosis caused by oxidative stress (168). This genomic region shares synteny with

rat Chr. 13, where a blood pressure QTL was mapped (46.4 - 112.6 Mb) in a previous study. *Glrx2* might have a role in regulating endothelial function presumably via mediating oxidative stress signaling. Similarly, the significant QTL on Chr. 2 (149.58-149.98 Mb) contains three genes not directly linked to vascular function regulation. However, this QTL is orthologous to rat QTL for blood pressure (20) and vascular growth and elastic tissue integrity (149, 199). Given the regions of shared synteny for cardiovascular traits between species, these suggestive QTL on Chrs. 1 and 2 are strong candidates for further validation studies, gene expression of candidate genes, and mechanistic studies of candidate genes.

At the suggestive level, 18 QTL for ACh Max were identified (Table 2.6). These suggestive QTL also contain several putative candidate genes for endothelial function (Table 2.7). Protein kinase (cAMP-dependent, catalytic) inhibitor gamma (*Pkig*) in the suggestive QTL on Chr. 2 (163.18-163.58 Mb) is involved in endothelial barrier dysfunction. Overexpression of this gene in endothelial cells reversed the barrier-enhancing effect of increased cAMP (172, 173). This suggestive QTL also overlaps with suggestive QTL for SNP IC_{50} and PE Max (Table 2.8), implying that this QTL contains gene(s), possibly *Pkig,* regulating vasomotor function of smooth muscle. Ubiquinol-cytochrome c reductase binding protein (*Uqcrb*) (suggestive QTL on Chr. 13) is known to regulate vascular endothelial growth factor receptor signaling and play a role in angiogenesis (39). Suggestive QTL on Chr. 17 contains heat shock protein 90kDa alpha, class B (*Hsp90ab1*). The stability of vascular endothelial growth factor (VEGF) receptors depends on *Hsp90ab1* function and inhibition of *Hsp90ab1* blocks the

proliferation and differentiation of endothelial cells (159). Given the stated roles of *Uqcrb* and *Hsp90ab1* in endothelial cell growth regulation, these genes might be attractive putative candidates for further studies.

Several genes residing in the suggestive QTL for ACh Max have been identified in human GWAS for cardiovascular traits. Mitochondrial ribosomal protein S22 (*Mrps22*) and Early B-cell factor 1 (*Ebf1*) in the QTL on Chrs. 9 and 11, respectively, were identified for blood pressure in humans (133, 185). QTL on Chr. 11 overlaps with QTL for the IC_{50} for SNP. Cell division cycle 5-like (*Cdc5l*) and mitochondrial ribosomal protein L14 (*Mrpl14*) on Chr. 17 have been identified in human GWAS for large artery ischemic stroke and circulating vascular endothelial growth factor, respectively (56, 120). An allelic effect of melanocortin 4 receptor (*Mc4r*; on Chr. 18) on blood pressure was also reported (178). For ACh IC_{50}, 5 QTL were found at the suggestive level. Though none of genes located in these QTL have known functions associated with sensitivity to ACh, some of them reportedly contribute to the vessel growth process, such as Sortilin-related receptor, L(DLR Class) A repeats containing (*Sorl1*) (293), Phosphatase and actin regulator 1 (*Phactr1*) (4) and Sirtuin 5 (*Sirt5*) (63). These genes have been identified in human GWAS for CVD-related traits (60, 63, 171). Based on conjunction with findings from human studies, some of our suggestive QTL would be confirmed with additional genomic analyses with more diverse populations.

While many QTL for vasoreactivity responses to SNP, PE and KCl were above the suggestive level, the majority of those QTL did not reach at the significant level (Table 2.8). One notable exception is a significant QTL for KCl IC_{50} on X chromosome.

This QTL contains several genes, all of which, however, have not been functionally linked to vascular regulation. None of those genes have been found in human GWAS or rat QTL related to cardiovascular traits. In a rat consomic panel, several chromosome consomic rat strains had different SNP IC_{50}, PE Max, PE EC50 compared to their parental inbred rat strains (150, 151). For example, chromosome 16 consomic rat strain and Brown Norway inbred rat strain had different aortic SNP IC_{50} compared with Dahl salt sensitive parental inbred rat strain (150). Maximal contractile responses to PE also varied by about three-fold among several consomic and inbred parental strains of rats (151). A handful of candidate gene studies have proposed a few genes, e.g. guanylate cyclase α-1 (34), smoothelin-like protein 1 (284), WNK lysine deficient protein kinase 1 (15), as regulators of smooth muscle vasomotor function. However, these genes were not found in our QTL, implying that there would be uncharacterized genetic factor(s) responsible for regulating smooth muscle responses to SNP, PE or KCl. Although strong candidate genes were not proposed by the present study, our result supports the hypothesis that smooth muscle vasomotor function is regulated by genetic factors and provides several suggestive QTL that would be valuable to explore further.

For the success of inbred strain GWAS studies, several factors were suggested to be considered; the number of inbred strains and genetic markers and the statistical algorithms. Wang et al. suggested that 30 strains or more would be recommended to have acceptable power for a trait having ~30 % genetic effect contributing to total variance (272). However, the intra-calss correlations (*rI*) in the present study are relatively high (Figs. 2.3 to 2.6); for example, 0.50 for ACh Max. Furthermore,

sequencing capability and single nucleotide polymorphism datasets have been dramatically increased (84, 287) and GWAS using larger single nucleotide polymorphism datasets with fewer than 30 strains have been successfully used in association studies for various complex traits (17, 118, 220). EMMA, which was utilized to perform GWAS in the present study, uses linear mixed models that provides increased statistical power as well (141). Thus, we believe that 26 inbred strains were sufficient to detect major single nucleotide polymorphism effect on endothelium-dependent vasorelaxation in the present study.

Numerous studies have established sex differences in vascular function (30, 186, 250, 276). For example, sex steroid hormones, such as estrogens and their receptors, are known to control endothelium-dependent vasorelaxation (186). Srivastava et al. found different genomic loci responsible for atherosclerosis between male and female mice (250). These previous data raise the possibility that the genetic basis for regulating endothelial function would be different between sexes. Sex differences were not considered in the present study, inclusion of females in the future studies should be considered in the future.

In summary, we found a wide range of differences in intrinsic vasoreactivity, mainly endothelium-dependent vasorelaxation, in aortas from 27 inbred mouse strains. These strain-dependent differences enabled us to perform GWAS with a dense single nucleotide polymorphism panel to identify QTL associated with intrinsic vascular function. In particular, GWAS for endothelium-dependent vasorelaxation revealed 4 significant and 18 suggestive QTL on several chromosomes and, these QTL contain a

few putative candidate genes which might play a role in regulating endothelial function. Even though further independent studies are necessary to replicate and refine our findings, the present study provides the first step toward comprehensive identification of genetic factors for complex endothelial function in a large genomic scale. Future studies may include linkage/association studies using more genetically diverse lines, such as Hybrid Mouse Diversity Panel (14) and Collaborative Cross (9), to increase both statistical and detection power with other comprehensive gene expression profiling approaches, e.g. RNA sequencing and expression QTL (3), to reproduce and refine our findings.

3. INTERACTION OF GENETIC BACKGROUND AND EXERCISE TRAINING INTENSITY ON ENDOTHELIAL FUNCTION IN MOUSE AORTA

3.1. Introduction

Impaired endothelial function is a fundamental component of the pathogenesis of cardiovascular disease. The endothelium plays an important role in maintaining vascular health via synthesis of various vasoactive mediators such as nitric oxide (NO), prostacyclin and endothelium-derived hyperpolarizing factors (188). Exercise training is generally known to improve endothelial function (58, 119, 137, 144). This beneficial effect of exercise training has been associated with increased expression of endothelial nitric oxide synthase (eNOS) (240), enhanced production and bioavailability of NO (231) and improved endothelium-dependent vasorelaxation (194). However, these findings are inconsistent. For example, Green et al. demonstrated a wide range of changes in flow-mediated dilation (FMD), a surrogate for endothelial function, in response to exercise training, including individuals who had no or negative changes in endothelial function following exercise training (96). The authors noted that greater training-induced changes in endothelial function were associated with lower initial cardiopulmonary fitness level and baseline endothelial function. Limited evidence also suggests that endothelial function is genetically influenced. In humans, the estimated heritability of FMD ranges from 0.14 to 0.44 (13, 122, 255, 290). Mouse strain

differences in endothelium-dependent vasorelaxation also have been reported (41, 233). For endothelial responses to exercise training, changes in FMD are more highly correlated in monozygotic than dizygotic twins (123). These data support the idea that responses to exercise training are also partially regulated by genetic factors.

The benefits from exercise training on the cardiovascular system, including endothelial function, appear to be dependent on the training intensity. Clinical trials and animal studies reported greater cardioprotective effects on VO_2max, blood pressure and glucose control after high intensity training (75 to 90 % of VO_2max) compared to moderate intensity training (< 70% of VO_2max) (117, 129, 144, 257). Conversely, several studies reported that high intensity training exerted similar effects as moderate intensity training, or no effects, on cardiovascular health (45, 106, 228). There is also some evidence linking high intensity exercise to negative outcomes (12, 130, 273). The effects of exercise training intensity on endothelial function are also inconsistent. While greater improvements in endothelial function in response to high intensity training compared to moderate intensity training have been observed in both humans and rats (114, 195, 262), others have reported no differences between moderate and high intensity training on endothelial function (144, 222). These inconsistent effects of training intensity might be due to the heterogeneity in age, sex and baseline health status (16, 93, 114, 144, 222, 262). However, the contribution of genetic background to these heterogeneous responses has not been formally considered.

Therefore, this study aimed to characterize the genetic contribution to endothelial adaptation to exercise training and to determine the interactive effect between genetic

background and training intensity on endothelial function. First, a strain survey for the effect of traditional exercise training on endothelium-dependent vasorelaxation was conducted in isolated thoracic aortas from 20 different inbred mouse strains. Then, four inbred mouse strains were chosen based on the strain survey and aortic endothelial responses to two different intensities (high *vs.* moderate) of exercise training were examined. It is hypothesized that endothelial adaptations to exercise training are variable among inbred mouse strains and these variable adaptations are dependent on training intensity.

3.2. Methods

3.2.1. Animals

All procedures adhered to the established National Institutes of Health guidelines for the care and use of laboratory animals and were approved by the Institutional Animal Care and Use Committee at Texas A&M University. First, seven-week old male mice from the following 20 inbred strains were utilized for the strain survey for vascular responses to traditional moderate intensity exercise training (n=4-8/strain): 129S1/SvImJ, 129X1/SvJ, A/J, BALB/cByJ, C57BL/6J, C57BR/cdJ, C58/J, CBA/J, CE/J, FVB/NJ, I/LnJ, LG/J, LP/J, MA/MyJ, NON/LtJ, NZO/HiLtJ, PL/J, PWD/PhJ, SJL/J, and SM/J. These inbred strains are a subset of 27 inbred mouse strains studied in the previous section. Then, four inbred strains, C57BL/6J (B6), 129S1/SvImJ (129S1), SJL/J (SJL), and NON/ShiLtJ (NON), were chosen based on the strain survey. Seven-week old male mice from each strain were randomly assigned to one of two exercise groups: exercise

training with continuous running at moderate intensity for 4 weeks (MOD, n = 6) or exercise training with interval running at high intensity for 4 weeks (HIT, n = 6). All mice were purchased from Jackson Laboratories. Upon arrival, all mice were given one week to acclimatize to their new environment. All mice were allowed food and water ad libitum and maintained on a 12:12-h light-dark cycle that initiated at 6:00 AM in the animal housing facility at Texas A&M University. Body weights were collected once a week throughout the study. Mice from the previous section were utilized as sedentary control mice (SED) to be compared with trained mice within the same strain in the present section.

3.2.2. Exercise performance test and exercise training

All mice (8-week old) were familiarized to treadmill running (10 min/d) for two days on a six-lane motorized rodent treadmill (Columbus Instruments, Columbus, OH). Each mouse then completed two exercise performance tests separated by 48 hours on the treadmill as described previously (50, 180). Briefly, the treadmill was started at 9 m/min at 0° grade for 9 minutes as a warm-up. The grade was then increased 5° every 9 minutes up to a final grade of 15° and speed was increased 2.5 m/min from a starting speed of 10 m/min every three minutes until exhaustion. Exhaustion was defined as an inability to maintain running in spite of repeated contact with the electric grid and manual stimulation. At exhaustion, each mouse was immediately removed from the treadmill and returned to its home cage. The average for two tests was used to calculate running speed for their training.

Traditional moderate intensity exercise training consisted of continuous running at 65% of maximal speed for 60 minutes a day as previously described (180). In 4 selected inbred strains, MOD mice performed continuous running at a 65% of maximal speed for ~70 minutes a day. HIT mice performed 6 sets of 8 minute-running at 85% of maximal speed followed by 2 minute-active rest at ~50% of maximal speed each session. All mice were trained 5 days/wk at a 10° incline on the treadmill for 4 weeks at ambient temperature (~24° C).

3.2.3. Tissue harvest and aortic ring experiments

Approximately 48 h after the final exercise bout, mice were weighed and anesthetized by intraperitoneal injection of a cocktail of ketamine (80 mg/kg) and xylazine (5 mg/kg). Subsequently, thoracic aortas were dissected and connective tissue was carefully removed in ice-cold physiological saline solution (in mmol/l: 118.3 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 25 $NaHCO_3$, and 5.5 glucose, pH 7.4) under a microscope. Then aortas were cut into 2 mm ring segments of equal length. Each ring segment was suspended in organ chamber of 610M Multi Chamber Myograph System (Danish Myo Technology, Denmark) filled with 8 ml of oxygenated (95% O_2, 5% CO_2) physiological saline solution and allowed to equilibrate at 37˚C for at least 30 minutes. Aortic rings were stretched to the resting tension (9 to 12 mN), which was determined by the tension-force assessment in response to 25 mM of potassium chloride (KCl). Then cumulative concentration-response curves to phenylephrine (PE: a selective α_1-adrenergic receptor agonist, 10^{-9} to 10^{-5} M) and KCl (a membrane

depolarizing agent, 5 to 100 mM) were generated to assess contractile function of aortic rings, while cumulative concentration-response curves to acetylcholine (ACh, muscarinic receptor agonist) and sodium nitroprusside (SNP, nitric oxide donor) (10^{-9} to 10^{-5} M) were generated to assess endothelium-dependent and -independent vasorelaxation, respectively. Concentration-response curves to ACh and SNP were generated after the ring was pre-constricted to 70% of maximum with PE. Doses were added after the response curve reached a plateau from the previous dose. Unused segments of thoracic aorta were snap-frozen in liquid nitrogen and stored for gene expression profiling analysis. Gastrocnemius muscles were collected, cut in half and stored for molecular analyses. All collected tissues were stored at -80℃.

3.2.4. Oxidative enzyme activity

Citrate synthase (CS) and succinate dehydrogenase (SDH) enzyme activity were measured in gastrocnemius muscles from SED, MOD and HIT mice as exercise training markers. A half of gastrocnemius muscle was placed in 20 volumes of ice-cold sucrose muscle homogenization buffer (20 mM Tris, 40 mM KCl, 2 mM EGTA, 250 mM Sucrose, pH was adjusted to 7.4) and homogenized using the FastPrep® -24 (MP Biomedicals, Santa Ana, CA). Homogenates were centrifuged at 600g for 10 min at 4° C. The supernatants were collected and total protein concentration was measured with BCA protein assay reagent and pre-diluted BSA standards (Thermo scientific, Waltham, MA) on Nanodrop 2000 (Thermo Scientific, Waltham, MA). Then citrate synthase and succinate dehydrogenase activity in the protein samples were assessed on the Genesys

10 UV spectrophotometer (Thermo scientific, USA) following a previously published protocol (249). Briefly, for CS enzyme activity, 300 µl of distilled water, 500 µl of Tris (200mM, pH 8.0) with Triton X-100 [0.2%(vol/vol)], 100 µl of 5,5'-dithiobis-(2-nitrobenzoic acid) (1 mM), 30 µl of Acetyl CoA (10 mM) and 20 µl of muscle homogenates were added to a 1-ml cuvette and the baseline activity at 412 nm was measured for 3 mins. Then, 50 µl of oxaloacetate (10 mM) was added and the absorbance change at 412 nm was measured for 3 mins. For SDH enzyme activity, 661 µl of distilled water, 50 µl potassium phosphate buffer (500 mM, pH 7.5), 20 µl of fatty acid-free bovine serum albumin (50 mg/ml), 30 µl of potassium cyanide (10 mM), 50 µl of succinate (400 mM), 145 µl of 2,6-dichlorophenol indophenol, and 40 µl of muscle homogenates were collected in a 1-ml cuvette and incubated at 37° C for 8 minutes. The baseline activity was measured at 600 nm for 3 mins. After starting the reaction by adding 4 µl of decylubiquinone (12.5 mM), the absorbance change at 600 nm was recorded for 3 mins. The molar extinction coefficients of 13.6 and 19.1 mM^{-1} cm^{-1} were used for CS and SDH enzyme activity calculation, respectively. All assays were carried out at room temperature unless specified. CS from porcine heart (Sigma-Aldrich, St. Louis, MO) was used as a standard for CS assay calibration.

3.2.5. RNA isolation, cDNA synthesis and PCR array

Frozen aortas from SED, MOD and HIT of three inbred strains (B6, SJL and NON) were homogenized and total RNA was isolated utilizing RNeasy fibrous tissue mini kit (Qiagen, Valencia, CA) according to the manufacturer's instruction. Aortas of

129S1 mice were excluded due to the similar vasomotor responses to exercise training as B6 mice. The quantity and quality of total RNA were determined by Nanodrop 2000 (Thermo Scientific, Waltham, MA) and BioAnalyzer 2100 (Agilent Technologies, Santa Clara, CA), respectively. RNA (500 ng) having an integrity number ≥ 7.5 were transcribed into cDNA using RT^2 First Strand cDNA Synthesis kit (Qiagen, Valencia, CA) following the manufacturer's manual. Synthesized cDNA were stored overnight at -20℃.

Gene expression profiling specific for mouse endothelial cell biology (Cat. No. PAMM-015Z) was conducted using RT^2 profiler PCR array (Qiagen, Valencia, CA) on a 96-well format. Each plate consists of 84 key genes associated with endothelial cell biology, 5 housekeeping genes, 1 mouse genomic DNA control, 3 reverse transcriptase control and 3 positive PCR controls. The complete list of genes can be found at the manufacturer's homepage (http://www.sabiosciences.com/rt_pcr_product/HTML/PAMM-015Z.html). The Real-Time PCR Array was performed as indicated in the user manual with RT^2 SYBR Green ROX qPCR Mastermix (Qiagen, Valencia, CA) using the StepOne Plus (Applied Biosystem, Waltham, CA). Expression levels for all genes were normalized with the geometric mean of five housekeeping genes, and the relative gene expression level was determined using the web-based data analysis software provided by Qiagen (http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php). This web-based software is designed to facilitate $2^{-\Delta\Delta C}{}_T$ calculation for PCR array data (170).

With results from PCR array, overrepresentation analysis was conducted in the Ingenuity Pathway Analysis (IPA; Ingenuity Systems, Redwood City, CA,

www.Ingenuity.com). Genes differentially expressed by exercise training ($P < 0.05$ compared to SED within the same strain) were queried against the pathway gene sets available in the IPA Knowledge Base to identify canonical pathways and molecular functions in which genes differentially expressed in each group/strain are involved. The core analysis was performed with a specific choice of 'mouse' for species and 'endothelial cell' for tissues/cells.

3.2.6. Nitrotyrosine Enzyme-Linked Immunoabsorbent Assay (ELISA)

Because nitrotyrosine is a product of protein tyrosine nitration resulting from oxidative damage to proteins by peroxynitrite, we measured abundance of nitrotyrosine in skeletal muscle from SED, MOD and HIT mice as an oxidative stress marker. Another half of the gastrocnemius muscle was homogenized and total protein was extracted using Cell Extraction Buffer (Invitrogen, Waltham, MA). Total protein concentration was measured as described above. The abundance of nitrotyrosine in the protein samples were measured using a 3-Nitrotyrosine ELISA kit (Abcam, Cambridge, MA) on the DTX800 Multi-mode microplate reader (Beckman Coulter, Brea, CA) according to the manufacturer's instruction.

3.2.7. Statistical analysis

Values are presented as mean ± SE. Percent vasocontractile responses (%) were calculated for PE and KCl as $[(D_P - D_B)/D_B] \times 100$, where '$D_P$' is the maximal force generated by a given specific dose and 'D_B' is the baseline force. Percent vasorelaxation

responses for ACh and SNP were calculated as $[(D_P - D_D)/(D_P - D_B)] \times 100$, where '$D_P$' is the maximal force pre-generated by PE, 'D_D' is the lowest force generated at a given dose of ACh or SNP and 'D_B' is the baseline force. The $\log_{10}$ of half maximal effective concentration (EC_{50}) for vasoconstriction responses and half maximal inhibitory concentration (IC_{50}) for vasorelaxation responses were calculated using absolute values (mN) from cumulative concentration-response curves using Prism 6 (GraphPad Software, La Jolla, CA) as the indicator for the sensitivity to a vasoactive agent.

Differences in maximal responses to vasoactive agents and EC_{50}/IC_{50} between traditional exercise trained mice and sedentary mice within each strain were analyzed using a Student's *t* test. For data from the second phase of study, differences in cumulative concentration-response curves across groups within a strain were compared using One-way ANOVA with repeated measures. Differences in body weight, mitochondrial enzyme activities, EC_{50} or IC_{50}, relative gene expression, and nitrotyrosine abundance across SED groups of each strain or groups within a strain were analyzed using One-way ANOVA followed by Tukey post-hoc test. Two-way ANOVA was conducted with strain-by-training mode to examine the interaction between strain and exercise training mode. Intrinsic differences at sedentary state between inbred strains were compared to SED of B6. All statistics were performed using SPSS 22 (IBM, Armonk, NY). Statistical significance was set at $P < 0.05$.

3.3. Results

To investigate genetic contribution to vascular (mainly endothelial) responses to exercise training in mice, a strain survey for vasoreactivity was performed in isolated thoracic aortas from 20 inbred mouse strains after 4 weeks of traditional moderate intensity exercise training. Figs. 3.1 to 3.4 illustrate the results of the strain survey of vascular responses to traditional exercise training in isolated thoracic aortas from 20 inbred strains. Overall, traditional exercise training had no effect on aortic endothelium-dependent vasorelaxation to ACh in most of inbred mouse strains (Fig. 3.1). Only aortic rings from NON/LtJ, A/J and CE/J showed greater maximal relaxation responses (%) to ACh (ACh Max) or IC_{50} for ACh (ACh IC_{50}) after traditional exercise training compared with sedentary mice within the same strain. The IC_{50} for SNP (SNP IC_{50}) in aortic rings was not affected by traditional exercise training in any strains, compared to sedentary mice of the same strain (Fig. 3.2). All aortic rings were 100% relaxed at SNP concentrations of 10^{-6} to 3×10^{-6} M, hence maximal responses to SNP were the same (data not shown). For vasoconstrictor agents, the majority of aortic rings from 20 inbred mouse strains, with only few exceptions, had similar contractile responses to PE and KCl between traditional exercise trained mice and sedentary mice within a strain (Figs. 3.3 and 3.4). Based on the result of endothelium-dependent vasorelaxation to ACh (Fig. 3.1), four inbred mouse strains (129S1, B6, SJL, and NON) were chosen to examine endothelial responses to two different training intensities.

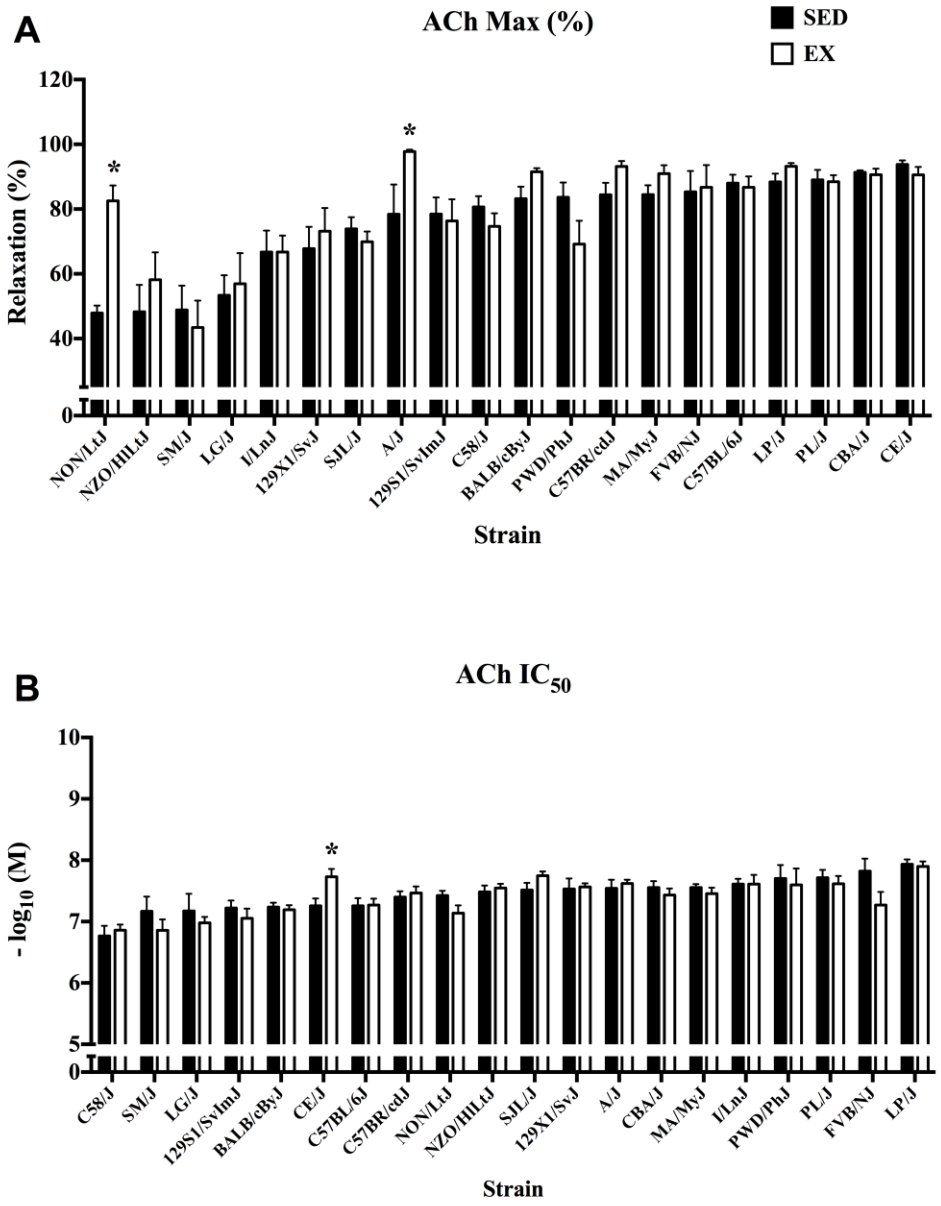

Figure 3.1. Strain survey for the effect of traditional exercise training on (A) maximal relaxation responses (%) to acetylcholine (ACh Max) and (B) the half maximal inhibitory concentration in responses to ACh (ACh IC_{50}) in young male mice from 20 inbred strains. After traditional moderate intensity exercise training for 4 weeks, cumulative concentration-response curves to ACh (10^{-9} to 10^{-5} M) were assessed in isolated thoracic aortas. *, P <0.05 significantly different from SED within the same strain.

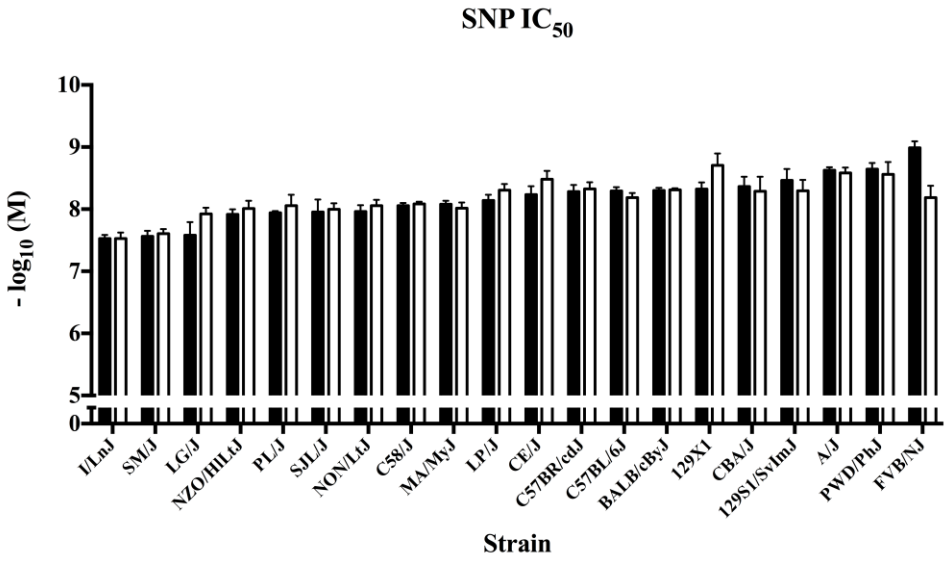

Figure 3.2. Strain survey for the effect of traditional exercise training on the half maximal inhibitory concentration in responses to sodium nitroprusside (SNP IC_{50}) in young male mice from 20 inbred strains. After traditional moderate intensity exercise training for 4 weeks, cumulative concentration-response curves to SNP (10^{-9} to 10^{-5} M) were assessed in isolated thoracic aortas. There was no difference in SNP IC_{50} between trained and sedentary mice within the same strain. Since all aortic rings were 100% relaxed at an SNP concentration of 10^{-6} to 3×10^{-6} M, maximal responses to SNP were not shown.

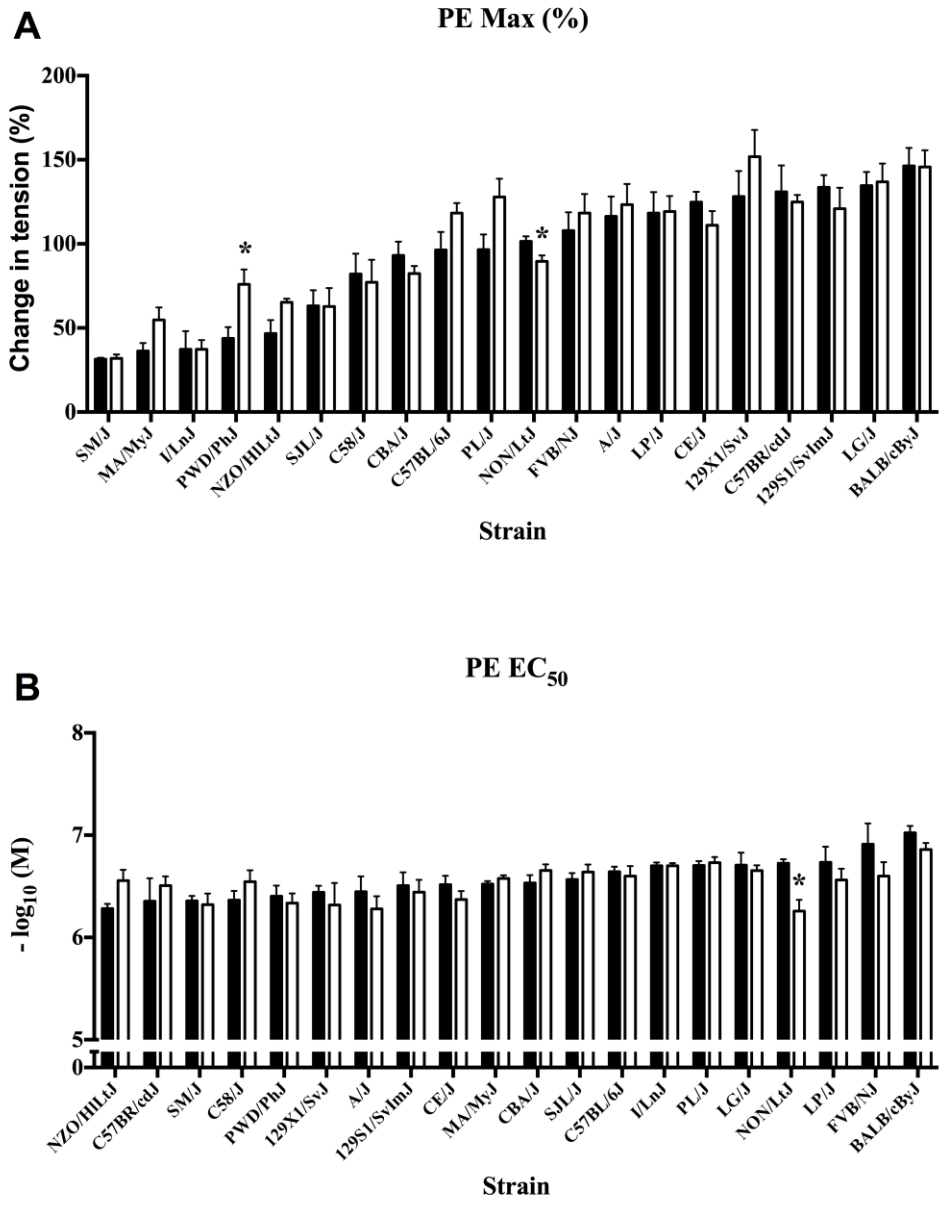

Figure 3.3. Strain survey for the effect of traditional exercise training on (A) maximal contractile responses (%) to phenylephrine (PE Max) and (B) the half maximal effective concentration in responses to PE (PE EC_{50}) in young male mice from 20 inbred strains. After traditional moderate intensity exercise training for 4 weeks, cumulative concentration-response curves to PE (10^{-9} to 10^{-5} M) were assessed in isolated thoracic aortas. *, P <0.05 significantly different from SED within the same strain.

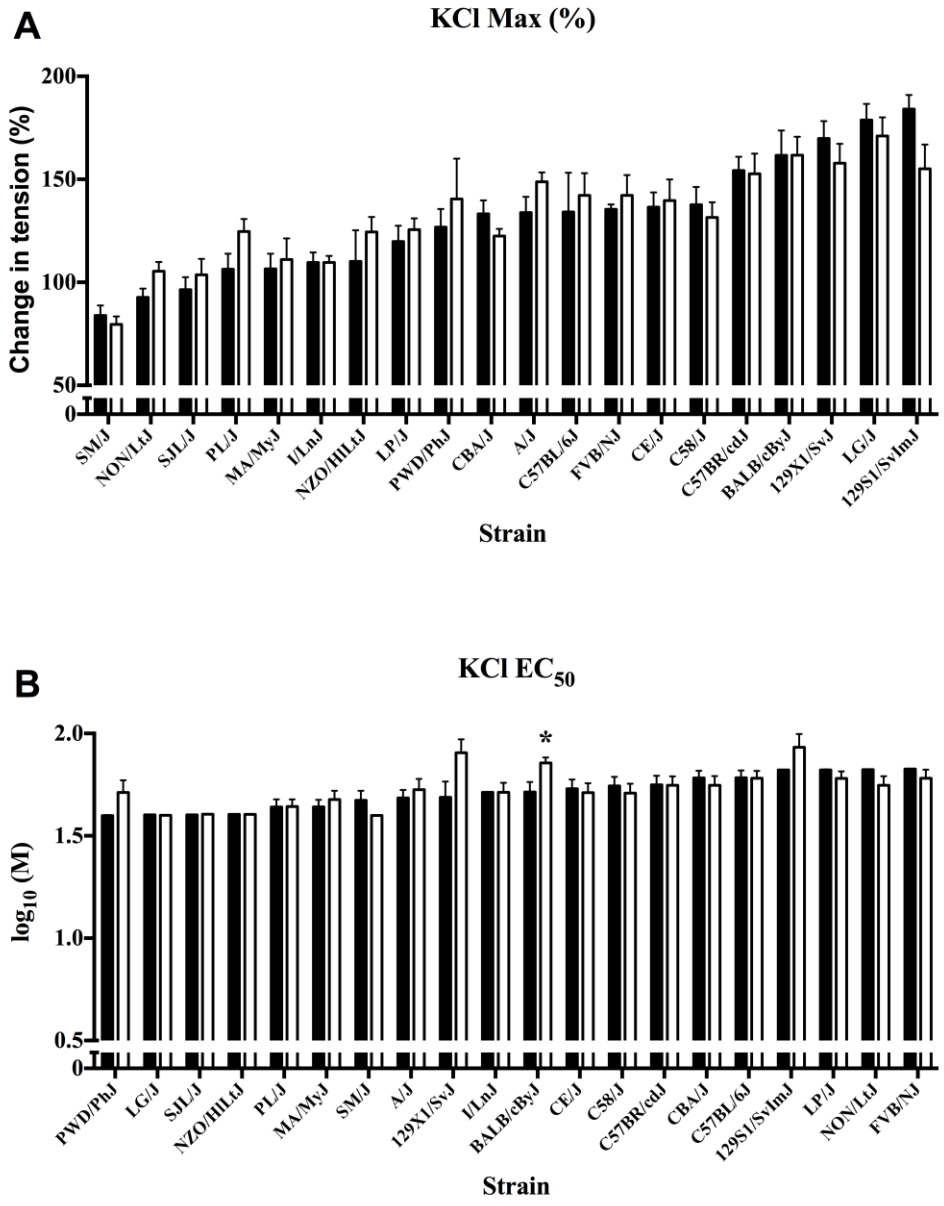

Figure 3.4. Strain survey for the effect of traditional exercise training on (A) maximal contractile responses (%) to potassium chloride (KCl Max) and (B) the half maximal effective concentration in responses to KCl (KCl EC_{50}) in young male mice from 20 inbred strains. After traditional moderate intensity exercise training for 4 weeks, cumulative concentration-response curves to KCl (5 to 100 mM) were assessed in isolated thoracic aortas. *, P <0.05 significantly different from SED within the same strain.

For four selected inbred strains, differences in pre-training body weight (pre-BW) and the change in BW (post minus pre-training) among groups or inbred strains are illustrated in Table 3.1. For pre-BW (g), NON had higher (30.6 ± 0.6) and SJL had lower (21.4 ± 0.4) pre-BW than B6 (23.5 ± 0.2). However, NON SED, as well as 129S1 SED, gained less BW than B6 SED after 4 weeks. In exercise trained mice, B6 HIT, SJL HIT and NON MOD showed smaller increases in BW compared with SED of the same strain. A significant interaction ($F = 8.36$, $P < 0.01$) between strain and training intensity was identified for the change in BW.

In order to assess exercise training efficacy, we measured citrate synthase (CS) and succinate dehydrogenase (SDH) enzyme activity in gastrocnemius muscles. Among SED mice, SJL had higher level of CS activity than B6 (Fig. 3.5A). In contrast, SDH activity was similar across SED groups of 4 inbred strains (Fig. 3.5B). In trained mice, MOD had higher CS and SDH activity in B6 and 129S1 compared to SED in the same strain. However, all HIT groups had similar CS and SDH activities compared with SED in the same strain. The interaction between strain and training intensity for SDH activity was significant ($F = 2.19$, $P = 0.03$), but not for CS activity ($F = 1.43$, $P = 0.19$). These results indicate that the effect of exercise training on oxidative enzyme activity in skeletal muscle is influenced by the interaction between inbred strain and training intensity.

Vasorelaxation responses to exercise training in isolated thoracic aortas from 4 inbred strains are summarized in Figs. 3.6 and 3.7. Both MOD and HIT groups of NON had greater endothelium-dependent vasorelaxation compared with NON SED.

Table 3.1. Pre-training body weight (BW) and the change in BW after exercise training in 4 inbred mouse strains

	C57BL/6J			129S1/SvImJ			SJL/J			NON/LtJ		
	SED	MOD	HIT	SED	MOD	HIT	SED	MOD	HIT	SED	MOD	HIT
Pre-training BW, g	24.1 ± 0.4	23.3 ± 0.5	23.3 ± 0.4	21.5 ± 0.7	21.7 ± 0.8	23.2 ± 0.6	21.6 ± 1.1	20.9 ± 0.7	21.6 ± 0.5	30.6 ± 1.2	32.1 ± 0.7	29.0 ± 0.7
Change in BW, g	4.2 ± 0.3	3.4 ± 0.4	2.4 ± 0.7*****	3.1 ± 0.1†	2.5 ± 0.3	2.9 ± 0.3	4.7 ± 0.4	3.6 ± 0.4	3.2 ± 0.3*****	3.0 ± 0.2†	0.1 ± 0.6*****	2.0 ± 0.4

Values are mean ± SE. *n* = 6 mice per group per strain. Pre-training BW, body weight before training at 8 wk-old; change in BW, body weight after training minus before training. *, $P < 0.05$ significantly different from SED within the same strain. †, $P < 0.05$ significantly different from SED of C57BL/6J.

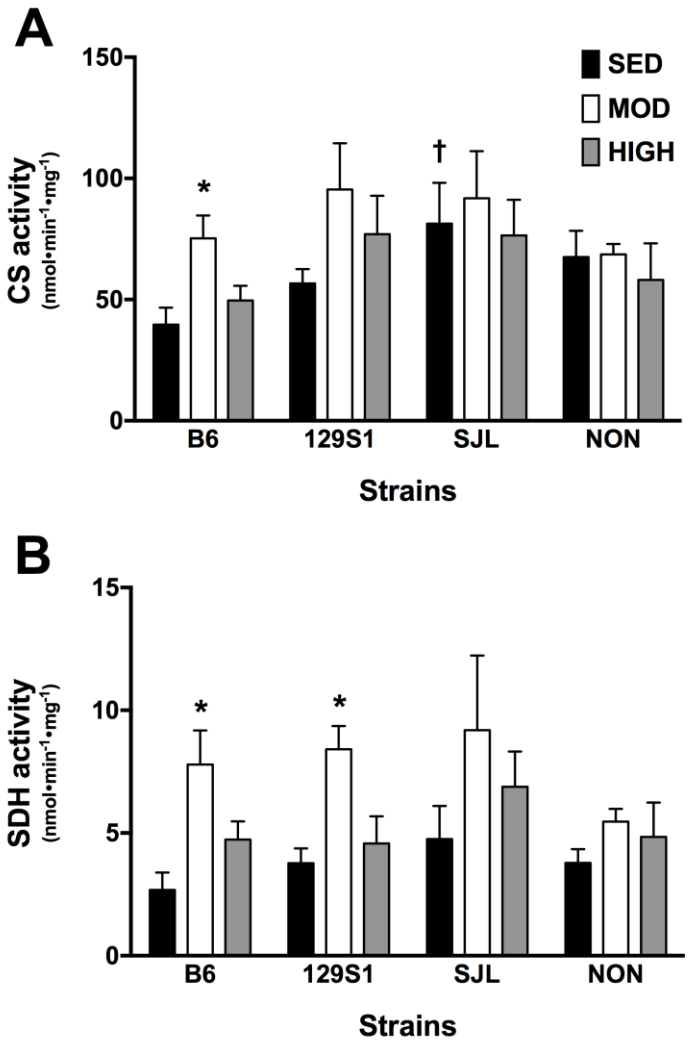

Figure 3.5. Effect of moderate-intensity continuous (MOD) and high-intensity interval training (HIT) on oxidative enzyme activity in gastrocnemius muscle from 4 inbred mouse strains. Eight-week old male mice (B6, 129S1, SJL, and NON) were trained with MOD or HIT for 4 weeks. (A) Responses of citrate synthase (CS) activity (nmol • min^{-1} • mg^{-1}) to two training intensities. (B) Responses of succinate dehydrogenase (SDH) activity (nmol • min^{-1} • mg^{-1}) to two exercise training intensities. Values are expressed as mean ± SE. *n* = 6 mice per group per strain. *, P <0.05 significantly different from SED within the same strain. †, P <0.05 significantly different from SED of B6.

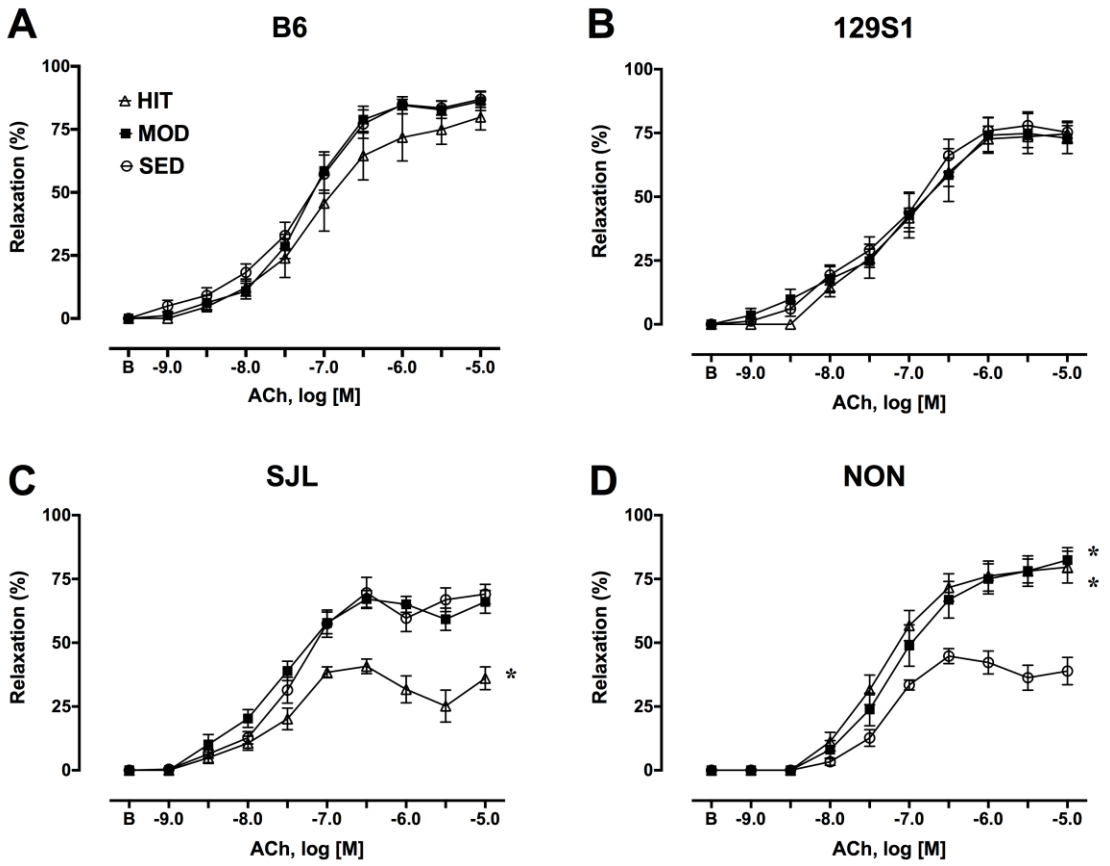

Figure 3.6. Effect of moderate-intensity continuous (MOD) and high-intensity interval training (HIT) on acetylcholine-induced endothelium-dependent relaxation in young male mice from 4 inbred strains. After exercise training with moderate intensity continuous running training (MOD) or high intensity interval training (HIT) for 4 weeks, cumulative concentration-response curves to acetylcholine (ACh, 10^{-9} to 10^{-5} M) were assessed in isolated thoracic aortas from 4 inbred strains, (A) B6, (B) 129S1, (C) SJL, and (D) NON. Cumulative concentration-response curves are expressed by percent relaxation (%). Values are expressed as mean ± SE. *n* = 6 mice per group per strain. *, P <0.05 significantly different from SED within the same strain.

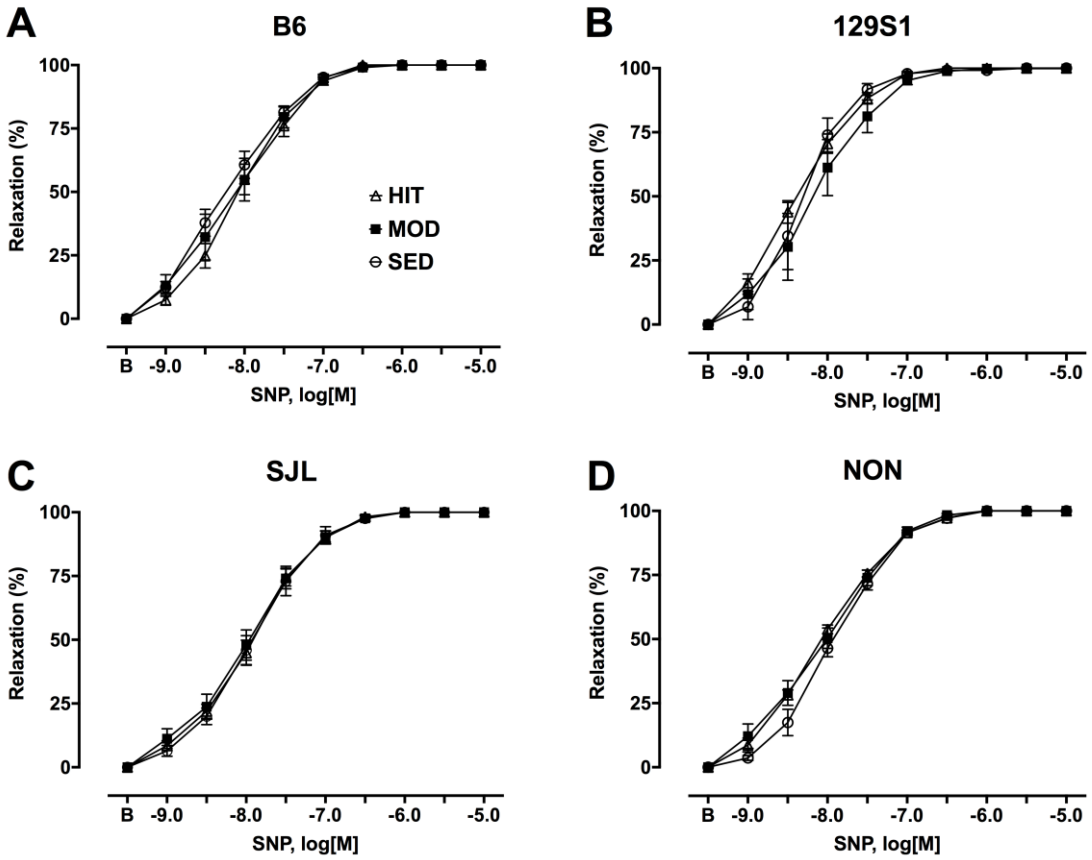

Figure 3.7. Effect of moderate-intensity continuous (MOD) and high-intensity interval training (HIT) on sodium nitroprusside-induced endothelium-independent vasorelaxation in young male mice from 4 inbred strains. After exercise training with moderate intensity continuous running training (MOD) or high intensity interval training (HIT) for 4 weeks, cumulative concentration-response curves to sodium nitroprusside (SNP, 10^{-9} to 10^{-5} M) were assessed in isolated thoracic aortas from 4 inbred strains, (A) B6, (B) 129S1, (C) SJL, and (D) NON. Cumulative concentration-response curves are expressed by percent relaxation (%). Values are expressed as mean ± SE. *n* = 6 mice per group per strain. SNP-induced endothelium-independent vasorelaxation was not different among groups within any of strains.

Table 3.2. IC_{50} and EC_{50} in cumulative concentration-response curves to vasoactive agents after exercise training in 4 inbred mouse strains

	C57BL/6J			129S1/SvImJ			SJL/J			NON/LtJ		
	SED	MOD	HIT	SED	MOD	HIT	SED	MOD	HIT	SED	MOD	HIT
ACh IC_{50} (log10)	- 7.26 ± 0.12	- 7.27 ± 0.10	- 7.04 ± 0.26	- 7.22 ± 0.12	- 7.05 ± 0.16	- 7.16 ± 0.09	- 7.51 ± 0.12	- 7.75 ± 0.07	- 7.88 ± 0.20	- 7.43 ± 0.07	- 7.14 ± 0.13	- 7.37 ± 0.08
SNP IC_{50} (log_{10})	- 8.29 ± 0.11	- 8.18 ± 0.19	- 8.09 ± 0.11	- 8.46 ± 0.16	- 8.30 ± 0.23	- 8.46 ± 0.08	- 7.96 ± 0.11	- 8.00 ± 0.11	- 7.96 ± 0.08	- 7.96 ± 0.06	- 8.06 ± 0.09	- 8.10 ± 0.03
PE EC_{50} (log_{10})	- 6.64 ± 0.05	- 6.60 ± 0.10	- 6.27 ± 0.13*****	- 6.51 ± 0.13	- 6.44 ± 0.12	- 6.48 ± 0.14	- 6.57 ± 0.06	- 6.64 ± 0.07	- 6.70 ± 0.11	- 6.73 ± 0.04	- 6.26 ± 0.11*****	- 5.97 ± 0.11*****
KCl EC_{50} (mM)	16.79 ± 1.63	16.86 ± 1.63	21.75 ± 2.05	15.07 ± 0.05	12.51 ± 2.45	16.57 ± 1.57	24.97 ± 0.03†	24.80 ± 0.05*****	24.91 ± 0.05	15.02 ± 0.07	18.39 ± 1.98	19.99 ± 2.02

Values are mean ± SE. *n* = 6 mice per group per strain. ACh, acetylcholine; SNP, sodium nitroprusside; PE, phenylephrine; KCl, potassium chloride; IC_{50}, half maximal inhibitory concentration in cumulative centration-response curve to ACh or SNP; EC_{50}, half maximal effective concentration in cumulative concentration-response curve to PE or KCl. *, $P < 0.05$ significantly different from SED within the same strain. †, $P < 0.05$ significantly different from SED of C57BL/6J.

In contrast, endothelium-dependent vasorelaxation in both exercise-trained groups (MOD, HIT) of B6 and 129S1 were not different from their respective SED mice. Interestingly, endothelium-dependent vasorelaxation was significantly impaired in SJL HIT compared with SJL SED (Fig. 3.6C). Sensitivity (IC_{50}) to ACh was not different across training groups for any of strains (Table 3.2). The interaction between strain and training intensity on endothelium-dependent vasorelaxation was significant ($F = 1.01$, $P < 0.01$). On the contrary, SNP-induced endothelium-independent vasorelaxation and SNP IC_{50} were not different among groups within each strain (Fig. 3.7 and Table 3.2). These results indicate that effect of exercise training on endothelium-dependent vasorelaxation, but not endothelium-independent relaxation, is influenced by the interaction between genetic background and training intensity.

For contractile responses to PE (Fig. 3.8), there were no differences between SED and exercise groups of B6, 129S1 and SJL. In contrast, both MOD and HIT of NON had decreased contractile responses to PE compared with NON SED. These decreased responses were accompanied by increased sensitivities (EC_{50}) to PE compared with SED (Table 3.2). Contractile responses to KCl were similar between SED and exercise groups in all strain (Fig. 3.9). Only SJL MOD had increased sensitivity (EC_{50}) to KCl compared with SJL SED (Table 3.2).

Because of the decreased endothelium-dependent vasorelaxation in SJL HIT, we measured the abundance of nitrotyrosine via ELISA in proteins extracted from gastrocnemius muscle to assess the effect of exercise training on oxidative stress (Fig. 3.10). NON had higher intrinsic (in the sedentary state) level of nitrotyrosine than B6.

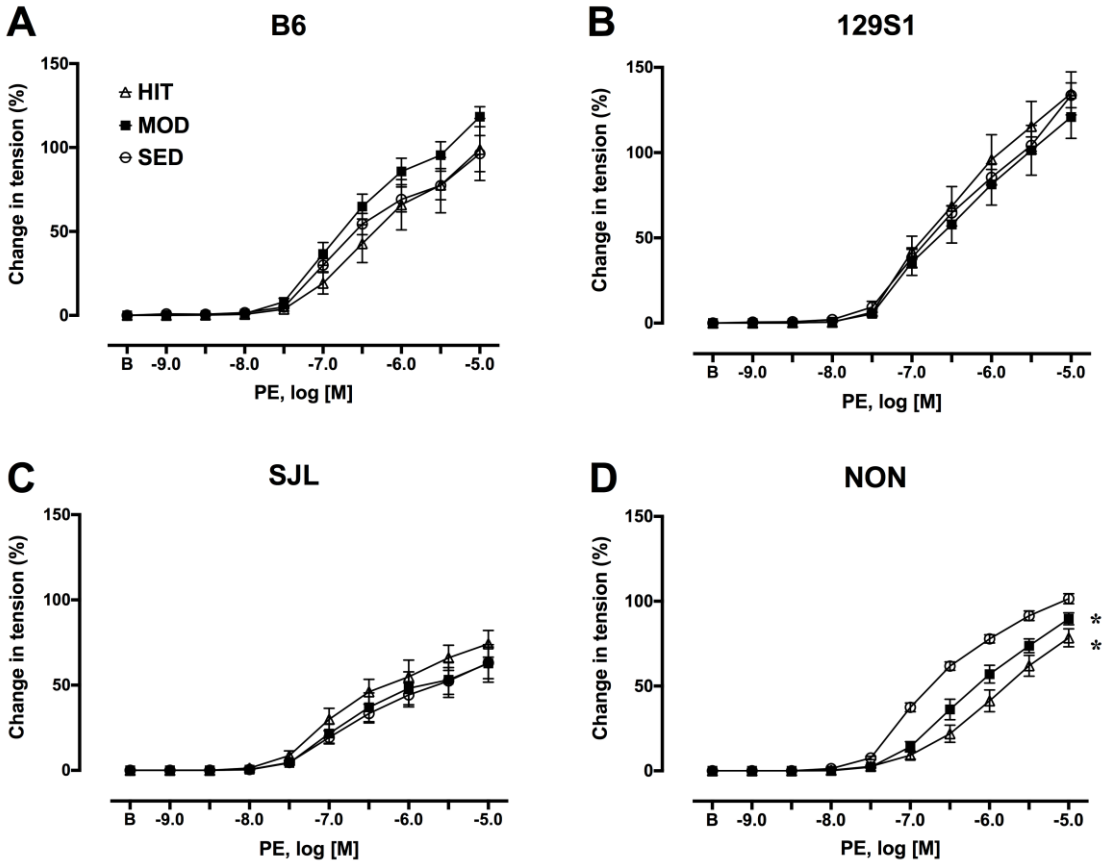

Figure 3.8. Effect of moderate-intensity continuous (MOD) and high-intensity interval training (HIT) on phenylephrine-induced contraction in young male mice from 4 inbred strains. After exercise training with moderate intensity continuous running training (MOD) or high intensity interval training (HIT) for 4 weeks, cumulative concentration-response curves to phenylephrine (PE, 10^{-9} to 10^{-5} M) were assessed in isolated thoracic aortas from 4 inbred strains, (A) B6, (B) 129S1, (C) SJL, and (D) NON. Cumulative concentration-response curves are expressed by change in tension (%). Values are expressed as mean ± SE. *n* = 6 mice per group per strain. *, P <0.05 significantly different from SED within the same strain.

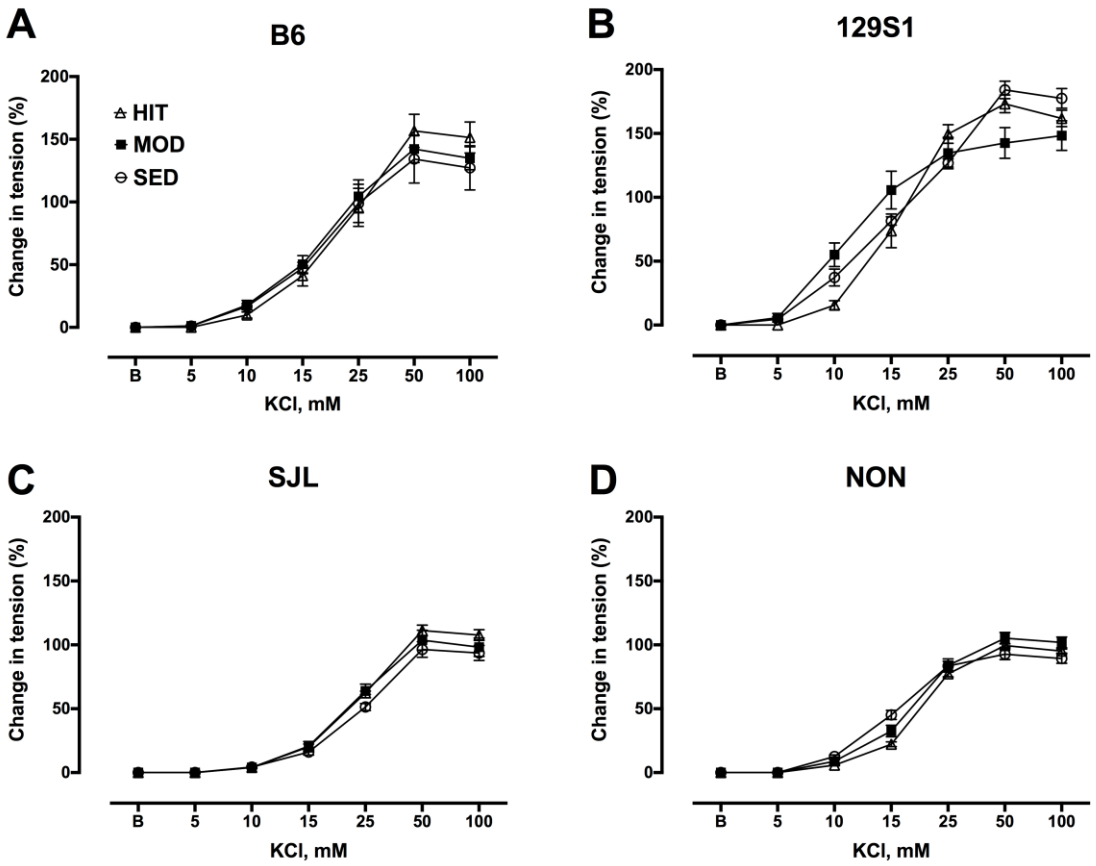

Figure 3.9. Effect of moderate-intensity continuous (MOD) and high-intensity interval training (HIT) on potassium chloride-induced contraction in young male mice from 4 inbred strains. After exercise training with moderate intensity continuous running training (MOD) or high intensity interval training (HIT) for 4 weeks, cumulative concentration-response curves to potassium chloride (KCl, 5 to 100 mM) were assessed in isolated thoracic aortas from 4 inbred strains, (A) B6, (B) 129S1, (C) SJL, and (D) NON. Cumulative concentration-response curves are expressed by change in tension (%). Values are expressed as mean ± SE. *n* = 6 mice per group per strain. KCl-induced contraction was not different among groups within any of strains.

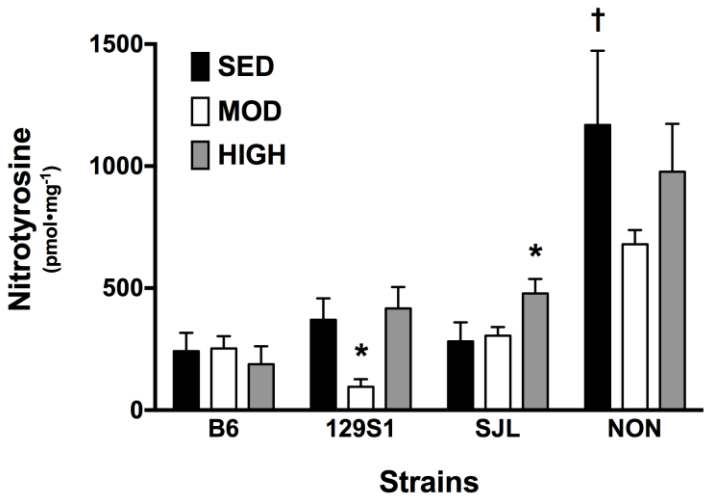

Figure 3.10. Effect of moderate-intensity continuous (MOD) and high-intensity interval training (HIT) on nitrotyrosine level in gastrocnemius muscle from 4 inbred mouse strains. 8-week old male mice were trained with moderate intensity continuous running training (MOD) or high intensity interval training (HIT) for 4 weeks. Values are expressed as mean ± SE. *n* = 6 mice per group per strain. *, P <0.05 significantly different from SED within the same strain. †, P <0.05 significantly different from SED of B6.

In exercise-trained mice, nitrotyrosine abundance was lower in 129S1 MOD compared with SED in the same strain. In SJL, nitrotyrosine abundance was higher in HIT than SED. A significant interaction ($F = 7.00$, $P < 0.01$) between strain and training intensity was found.

To determine which genes are differentially expressed by exercise training, we conducted expression profiling of endothelial cell biology-related genes by RT-qPCR. The pattern of gene expression changes in trained groups compared with SED in the same strain is visualized in Fig. 3.11 and the lists of genes differentially expressed by exercise training compared to SED within each strain ($p < 0.05$) are shown in Tables 3.3 and 3.4. For B6, only a few genes were differentially expressed (1 up- and 4 down-regulated) between MOD or HIT and SED. For SJL mice, 1 gene was up-regulated and 4 genes were down-regulated in MOD, while 9 genes were up-regulated and 2 genes were down-regulated in HIT (Table 3.3). NON MOD had 3 and 6 genes up- and down-regulated by exercise training, respectively. There were 28 genes (21 up-regulated and 7 down-regulated) differentially expressed in NON HIT (Table 3.4). There was very little overlap between genes differentially expressed in MOD and HIT within each strain or among strains. These data suggest that the influence of exercise training on transcriptional activation is both strain- and training intensity-dependent.

Gene expression profiling data derived from PCR array were imported into the IPA to identify biological pathways and molecular/cellular functions overrepresented with genes differentially expressed in exercise-trained groups. Only data for SJL HIT, NON MOD and NON HIT were analyzed because of limited changes in the other groups.

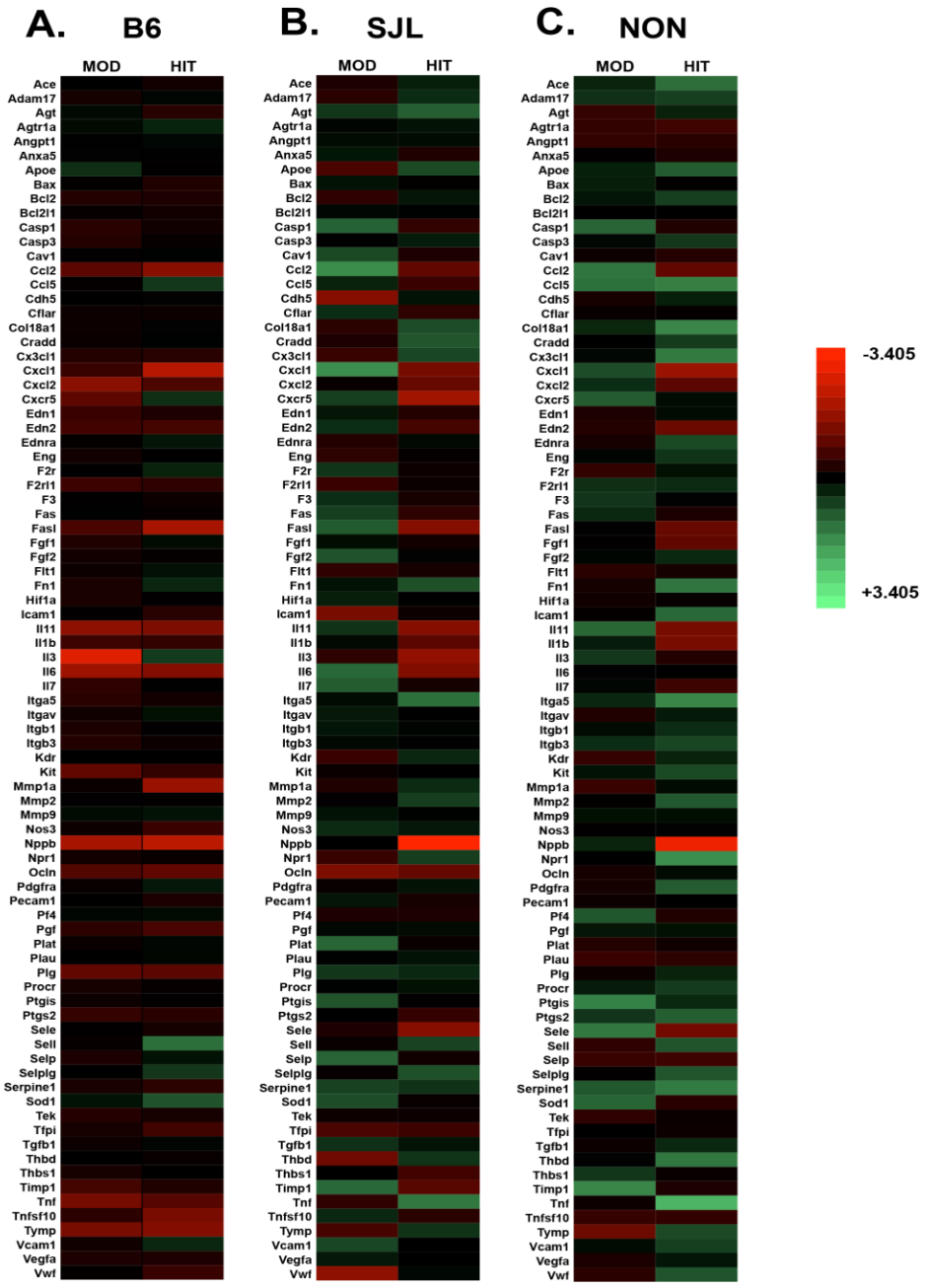

Figure 3.11. Heat-map of relative expression levels of 84 key genes associated with endothelial cell biology in trained mice compared with sedentary mice within the same strain. 129S1 strain was excluded for gene expression profiling due to similar results as B6. 8-week old male mice were trained with moderate intensity continuous running training (MOD) or high intensity interval training (HIT) for 4 weeks. Red indicates down-regulation, black indicates no change and green indicates up-regulation. n = 4 mice per group per strain.

Table 3.3. Genes differentially expressed by exercise training in thoracic aortas from B6 and SJL mice

Strain	Training	Symbol	Name	Fold regulation	*p* value
B6	MOD	***Down-regulated***			
		Vegfa	Vascular Endothelial Growth Factor A	-1.2235	0.006971
	HIT	***Up-regulated***			
		Sod1	Superoxide dismutase 1, soluble	1.8638	0.008099
		Down-regulated			
		Bax	BCL2-associated X protein	-1.2333	0.01693
		Ccl2	Chemokine (C-C motif) ligand 2	-2.8841	0.005821
		Pecam1	Platelet/endothelial cell adhesion molecule 1	-1.1998	0.021993
		Tnfsf10	Tumor necrosis factor (ligand) superfamily, member 10	-2.5176	0.003915
SJL	MOD	***Up-regulated***			
		Cxcl1	Chemokine (C-X-C motif) ligand 1	3.2444	0.002115
		Down-regulated			
		Adam17	A disintegrin and metallopeptidase domain 17	-1.3187	0.019273
		Cdh5	Cadherin 5	-2.8288	0.040645
		Kdr	Kinase insert domain protein receptor	-1.4956	0.036468
		Thbd	Thrombomodulin	-2.3078	0.04334
	HIT	***Up-regulated***			
		Adam17	A disintegrin and metallopeptidase domain 17	1.3688	0.008579
		Agt	Angiotensinogen	2.0555	0.022339
		Apoe	Apolipoprotein E	1.7175	0.000182
		Cradd	CASP2 and RIPK1 domain containing adaptor with death domain	1.9187	0.002729
		Cx3cl1	Chemokine (C-X3-C motif) ligand 1	1.68	0.01296
		Itga5	Integrin alpha 5	2.4118	0.000414
		Mmp2	Matrix metallopeptidase 2	1.5643	0.016875
		Npr1	Natriuretic peptide receptor 1	1.5639	0.008387
		Tnf	Tumor necrosis factor	2.6159	0.012139
		Down-regulated			
		Ccl2	Chemokine (C-C motif) ligand 2	-2.0397	0.04728
		Cflar	CASP8 and FADD-like apoptosis regulator	-1.3652	0.002045

Among 84 key genes associated with endothelial cell biology, genes significantly up- and down-regulated by exercise training ($P<0.05$, compared to SED within a strain) are listed.

Table 3.4. Genes differentially expressed by exercise training in thoracic aortas from NON mice

Strain	Training	Gene	Description	Fold regulation	*p* value
		Up-regulated			
		Ccl2	Chemokine (C-C motif) ligand 2	2.5446	0.042696
		Pf4	Platelet factor 4	1.9346	0.027056
		Ptgis	Prostaglandin I2 (prostacyclin) synthase	2.8793	0.039385
		Down-regulated			
	MOD	Agtr1a	Angiotensin II receptor, type 1a	-1.3952	0.018257
		Cdh5	Cadherin 5	-1.1755	0.040467
		Flt1	FMS-like tyrosine kinase 1	-1.3173	0.045257
		Kdr	Kinase insert domain protein receptor	-1.4242	0.013216
		Mmp1a	Matrix metallopeptidase 1a (interstitial collagenase)	-1.47	0.007821
		Tnfsf10	Tumor necrosis factor (ligand) superfamily, member 10	-1.4434	0.005505
		Up-regulated			
		Ace	Angiotensin I converting enzyme	2.3746	0.010898
		Adam17	A disintegrin and metallopeptidase domain 17	1.5966	0.008599
		Apoe	Apolipoprotein E	2.019	0.000432
		Casp3	Caspase 3	1.5061	0.0478
NON		Cdh5	Cadherin 5	1.2575	0.008199
		Col18a1	Collagen, type XVIII, alpha 1	3.0057	0.035492
		Cradd	CASP2 and RIPK1 domain containing adaptor with death domain	1.5422	0.001839
		Cx3cl1	Chemokine (C-X3-C motif) ligand 1	2.656	0.010743
		Ednra	Endothelin receptor type A	1.7285	0.014541
		Eng	Endoglin	1.4588	0.040187
	HIT	Fn1	Fibronectin 1	2.5669	0.004596
		Icam1	Intercellular adhesion molecule 1	2.2695	0.01039
		Itga5	Integrin alpha 5	2.9982	0.001567
		Kit	Kit oncogene	1.7288	0.043853
		Mmp2	Matrix metallopeptidase 2	1.9703	0.010397
		Npr1	Natriuretic peptide receptor 1	3.2579	0.004695
		Pdgfra	Platelet derived growth factor receptor, alpha	2.0388	0.011554
		Procr	Protein C receptor, endothelial	1.5567	0.037544
		Tgfb1	Transforming growth factor, beta 1	1.3271	0.03762
		Thbd	Thrombomodulin	2.6407	0.000892
		Vwf	Von Willebrand factor homolog	1.8964	0.018501

Table 3.4 Continued

Strain	Training	Gene	Description	Fold regulation	*p* value
		Down-regulated			
		Agtr1a	Angiotensin II receptor, type 1a	-1.5457	0.002137
		Cxcl1	Chemokine (C-X-C motif) ligand 1	-3.4433	0.022196
		Cxcl2	Chemokine (C-X-C motif) ligand 2	-1.9636	0.019817
		Edn2	Endothelin 2	-2.2056	0.039311
		Fgf1	Fibroblast growth factor 1	-2.0569	0.001005
		Sele	Selectin, endothelial cell	-2.3573	0.024778
		Tnfsf10	Tumor necrosis factor (ligand) superfamily, member 10	-1.3849	0.007946

Among 84 key genes associated with endothelial cell biology, genes significantly up- and down-regulated by exercise training ($P<0.05$, compared to SED within a strain) are listed.

Table 3.5. Top 3 canonical pathways for genes significantly altered by exercise training

Strain	Training	Description	*p* value	Genes
SJL	HIT	Agranulocyte Adhesion and Diapedesis	4.89E-04	Cx3cl1, Itga5, Mmp2, Tnf
		Granulocyte Adhesion and Diapedesis	6.12E-04	Cx3cl1, Itga5, Mmp2, Tnf
		FXR/RXR Activation	8.95E-04	Agt, Apoe, Tnf
NON	MOD	Hepatic Fibrosis / Hepatic Stellate Cell Activation	6.18E-04	Flt1, Kdr, Mmp1a, Tnfsf10
		VEGF Family Ligand-Receptor Interactions	3.08E-03	Flt1, Kdr
		Nitric Oxide Signaling in the Cardiovascular System	5.71E-03	Flt1, Kdr
	HIT	Agranulocyte Adhesion and Diapedesis	3.73E-06	Cdh5, Cx3cl1, Fn1, Icam1, Itga5, Mmp2, Cxcl2, Sele
		Granulocyte Adhesion and Diapedesis	6.73E-05	Cdh5, Cx3cl1, Fn1, Icam1, Itga5, Mmp2, Cxcl2, Sele
		Hepatic Fibrosis / Hepatic Stellate Cell Activation	9.86E-05	Col18a1, Ednra, Fn1, Icam1, Mmp2, Tgfb1, Cxcl2, Tnfsf10

Canonical pathways to which genes significantly altered by exercise training belong were identified by Ingenuity Pathway Analysis (IPA). Selection of top 3 canonical pathways was based on P-value which is a measure of the likelihood that the association between a set of genes and a given pathway is due to random chance. The *p* value is calculated by the right-tailed Fisher Exact Test in IPA. Data from the other groups/strains were excluded due to limited changes in both gene expression (Tables 3.3) and endothelial function after exercise training (Fig. 3.6).

Table 3.6. Top 3 molecular and cellular functions for genes significantly altered by exercise training

Strain	Training	Description	*p* value	Predicted
SJL	HIT	Production of reactive oxygen species	9.66E-04	Up
		Synthesis of nitric oxide	1.79E-03	Up
		Synthesis of phosphatidylinositol-3,4,5-triphosphate	1.72E-02	Up
NON	MOD	Differentiation of endothelial cells	5.39E-05	Down
		Endothelial cell development	2.98E-03	Up
		Proliferation of endothelial cells	3.06E-02	Up
	HIT	Adhesion of endothelial cells	9.09E-05	Down
		Adhesion of immune cells	1.01E-04	Down
		Migration of endothelial cells	1.27E-03	Down

Diseases or molecular functions with which genes significantly altered by exercise training are associate were identified by Ingenuity Pathway Analysis (IPA). Selection of top 3 functions was based on P-value calculated by the right-tailed Fisher Exact Test in IPA. Data from the other groups/strains were excluded due to limited changes in both gene expression (Tables 3.3) and endothelial function after exercise training (Fig. 3.6).

IPA identified several canonical pathways and molecular/cellular functions with which genes differentially expressed in exercise-trained groups are associated (Tables 3.5 and 3.6). Gene sets differentially expressed in HIT groups of SJL and NON were overrepresented in pathways related to inflammatory molecule adhesion and migration, while a gene cluster differentially expressed in NON MOD was enriched in pathways associated with vessel growth and NO signaling. For the overrepresented molecular and cellular functions, the greatest effects of HIT in SJL appeared to be related to molecular functions of reactive oxygen species (ROS) production, whereas the effects of HIT in NON involved molecular functions linked to cell adhesion and migration. The genes differentially expressed in NON MOD were associated with cell differentiation and proliferation.

3.4. Discussion

First, the effect of traditional exercise training on vasoreactivity was globally evaluated in thoracic aortas from 20 inbred mouse strains to characterize the effect of genetic background on endothelial responses to a commonly used exercise training program in mice. Then, in 4 selected inbred strains, vascular responses to two different intensities of exercise training in thoracic aortas were further assessed to determine the interactive effect between genetic background and training intensity on endothelial function. The main findings were: 1) traditional exercise training exerted subtle effects on endothelial function in the majority of inbred mouse strains; 2) intrinsic physiological markers of skeletal muscle in the sedentary state were variable across four selected

inbred strains; 3) there was a significant interaction between genetic background and training intensity on endothelial responses to exercise training; 4) endothelial gene expression profiles were different depending on both genetic background and training intensity.

It is generally known that exercise training can improve endothelial function (58, 119, 137, 145). However, in the present study, the strain survey revealed that endothelium-dependent vasorelaxation in aortic rings from 20 inbred mouse strains were similar between trained and sedentary groups after 4 weeks of traditional moderate intensity exercise training (Fig. 3.1). Although results are mixed, several previous studies have reported that exercise training had no impact on endothelium-dependent vasorelaxation in young normal populations (97, 203, 264). Green et al. proposed a possible explanation that the lack of benefit from exercise training might be due to training-induced vessel remodeling which may structurally normalize the responses to increased shear stress stimuli (97). This exercise-induced vascular remodeling, however, tends to occur after a long-term exercise training (typically ≥ 12 to16 weeks) (32, 137, 277), implying that this might not be the cause of the lack of responses to 4 weeks of traditional moderate intensity exercise training in the present study. Rather, given the feasibility that all mice were young (13 week-old) and thus were presumed to have normal baseline endothelial function, the intensity of traditional exercise training might not be sufficient to stimulate functional changes in endothelium. This possibility prompted us to investigate the effect of high intensity exercise training (HIT) on endothelial function in selected inbred mouse strains.

Among 4 selected inbred strains in the sedentary state, NON had impaired baseline endothelial function, whereas 129S1 and SJL had similar baseline endothelial function, compared to B6 (Fig. 2.3). NON had a significantly higher skeletal muscle nitrotyrosine, a marker of oxidative stress, in the sedentary state compared with B6 (Fig. 3.10). Increased oxidative stress can lead to reduction in nitric oxide (NO) bioavailability and subsequently reduced response to endothelium-dependent vasorelaxing agents (35). Additionally, results from gene expression profiling indicated that NON SED had moderately decreased expression in eNOS gene (Fold regulation = -1.48, P = 0.07) compared with B6 SED (data not shown). Although our data, as well as previous studies (219, 256), indicate that NON exhibits phenotypes associated with endothelial dysfunction, the underlying mechanism of their impaired endothelial function is not clear and further studies are required.

CS and SDH activity in skeletal muscle have been widely employed as markers for mitochondrial oxidative potential. In the present study, intrinsic CS activity varied across strains (Fig. 3.5A), suggesting a genetic contribution to intrinsic CS activity in mouse skeletal muscle. This finding is in agreement with Ratkevicius et al. in that there is a difference in intrinsic CS activity among inbred mouse strains (224). These authors identified a putative single nucleotide polymorphism (rs29358506) that contributed to the difference in intrinsic CS activity across 6 inbred mouse strains. We could not support their finding because all 4 strains we utilized in the present study have the same genotype for this polymorphism. For SDH activity, we found no difference among the 4

inbred strains (Fig. 3.5B) and thus do not have evidence of a genetic contribution to intrinsic SDH activity in this study.

It is well established that exercise training improves endothelial function (58, 119, 137, 145). We hypothesized that 4 week-exercise training would improve endothelial function and mice completing HIT would have augmented endothelial responses (57, 114). As expected, endothelium-dependent vasorelaxation was greater in aortas from MOD and HIT of NON compared with their SED (Fig. 3.6D). This enhanced endothelial function was accompanied by lower PE-induced vasoconstriction in those groups (Fig. 3.8D). NON SED had the lowest endothelial function among inbred strains (Fig. 2.3). Therefore, exercise training would be expected to improve endothelial function. Previous studies reported that exercise training increased expression of eNOS and SOD-1 gene, bioavailability of NO and the release of NO (231, 240). However, in the present study, eNOS or SOD-1 gene expression was not altered in the exercise groups (Table 3.4), nor was nitrotyrosine level in skeletal muscle (Fig. 3.10). Thus, exercise training-induced improvements in endothelial function in NON might be due to an increase in other vasorelaxation mechanisms rather than an increase in eNOS expression or NO bioavailability, for an example, increased prostacyclin synthase gene expression observed in NON MOD (Table 3.4). In contrast, markers of oxidative capacity in skeletal muscle were not different among exercise training and SED in NON (Fig. 3.5). Green et al. reported that exercise training-induced change in endothelial function did not correlate with the change in VO_2max (96). In the same sense, our results

provide evidence that endothelial function responses in exercise-trained mice are independent of the change in skeletal muscle oxidative capacity.

ACh-induced endothelium-dependent vasorelaxation was similar between MOD and HIT groups in aorta from NON mice. In many studies, HIT has been proposed as an effective alternative to the traditional aerobic training (MOD), inducing similar or even superior cardiovascular adaptations (114, 117, 129, 144, 195, 257, 262). Furthermore, similar or greater effects of HIT than MOD have been described for endothelial function in young subjects (114, 144, 222).

In the present study, endothelium-dependent vasorelaxation was not altered by any exercise training intensity in either B6 or 129S1. There are many factors, such as age, sex, training duration, exercise intensity, and genetic regulation, which can influence the effect of exercise training on endothelial function (57, 66, 96, 144). We matched age (7 wk-old at the beginning) and sex (male) of experimental groups to avoid some of these confounding effects. We also chose to train mice for 4 weeks because training duration of at least 4 weeks has been shown to induce an improvement in aortic endothelial function in healthy animals (57). However, other studies have reported that endothelial function is not improved by exercise training in young healthy humans and animals (95, 203, 264). At least three possible factors might explain this lack of exercise-induced improvements in endothelium-dependent vasorelaxation in B6 and 129S1. First, the training protocol, even HIT, might exert insufficient stimuli to change endothelial function in aortas from these strains. However, our training volume was sufficient to increase oxidative enzyme activity in skeletal muscle in both strains (Fig. 3.5). Secondly,

structural adaptation had already occurred before post-training measures were applied in these mice, which can normalize the effect of the increased vasorelaxing stimuli on vascular smooth muscle relaxation (96, 137). Unfortunately, we did not assess vessel size in the present study. Thirdly, the endothelial phenotype in those two strains might be near optimal levels, thus endothelial function may not be augmentable above the levels observed in the respective SED groups for each strain (66, 203). This latter explanation is supported by the small number of genes differentially expressed between SED and exercise-training groups in aorta from B6 mice (Table 3.3). The one notable exception is the increased expression of SOD-1, an antioxidant pathway gene that is commonly reported to increase with exercise training. Overall, our results in B6 are similar to those reported by Padilla et al. who found no differences in endothelial function and gene expression in conduit arteries from healthy pigs after 16 to 20 weeks of exercise training (203).

Although many previous studies have recommended HIT as a time-efficient training method to improve cardiovascular health, there is currently accumulating evidence in the literature that intense training can induce adverse effects on cardiovascular function. In the present study, endothelial function was not different in MOD, but was impaired in HIT compared to SED of SJL (Fig. 3.6C). Previous studies reported that intense exercise caused a decrease in eNOS expression and NO level in hearts of young rats (130) and induced platelet aggregation in young healthy humans, which may augment the risk of vascular thrombosis (273). Bergholm et al. found that vigorous aerobic training impaired endothelium-dependent vasodilation and decreased

antioxidant concentrations in young individuals (16). Also, high intensity exercise training induced an increase in plasma concentration of 8-hydroxy-2'-deoxyguanosine, a marker of oxidative stress to DNA, with no beneficial effect on endothelial function (93). In the present study, skeletal muscle nitrotyrosine level was higher in SJL HIT compared with SED and MOD groups (Fig. 3.10). The higher nitrotyrosine levels in HIT could reflect an overall higher oxidative stress in this group, which contribute to the impaired endothelial function observed in the aorta of the SJL HIT. Our finding supports the previously proposed concept that a threshold of training intensity exists beyond which ROS generation overrides the scavenging capabilities of antioxidant systems in the vasculature (66). Therefore, comparing the different effects of HIT on endothelial function between NON and SJL in the present study, the beneficial threshold of training intensity would be determined by genetic background.

SNP-induced endothelium-independent vasorelaxation was not changed by any of exercise training intensity in all four inbred strains (Fig. 3.7). This result is consistent with previous studies (114, 144, 264), demonstrating that vascular smooth muscle responses to nitric oxide (NO) are not generally influenced by exercise training in young animals.

We utilized expression profiling of a number of genes to study the influence of exercise training on endothelial cell-specific transcriptional activation and biological functions. Overall, the number of differentially expressed genes was proportional to the difference in endothelium-dependent vasorelaxation between SED and exercise-trained groups (Table 3.3 and 3.4). For example, only one gene was differentially expressed

between B6 SED and MOD groups, which had similar endothelium-dependent vasorelaxation. By comparison, NON HIT had significantly greater endothelium-dependent vasorelaxation compared with NON SED and 28 genes were differentially expressed. Furthermore, the number of genes differentially expressed in HIT was greater than MOD in all 3 strains and only a few genes overlapped between MOD and HIT. This observation is opposite to a previous study that moderate intensity endurance training produced greater transcriptional effects on isolated aortic endothelial cells from rats than interval sprint training (203). However, these authors also reported that interval sprint training had a greater effect on gene expression in skeletal muscle feed arteries than endurance exercise training. Thus, transcriptional responses to exercise training might be dependent on species as well as vessel characteristics and training intensity.

Differences in training intensity can influence not only the number of genes differentially expressed, but also the signaling pathways in which those genes are involved. For example, gene sets differentially expressed in HIT groups of SJL and NON generally involved pathways related to inflammatory molecule adhesion and migration, whereas a gene set differentially expressed in NON MOD belonged to growth and NO signaling-related pathways (Table 3.5). A previous study suggested that different intensity training programs exert shear stress on the vessel walls differently during exercise and that this yields differences in molecular responses (262). Similarly, Padilla et al. reported no overlap for gene networks influenced by endurance and interval sprint training for skeletal muscle feed arteries or rat aortic endothelial cells (202). Thus, our findings that the overrepresented canonical pathways diverged between MOD and

HIT further provide evidence for the intensity-specific pattern of training-induced transcriptional activation in aortic endothelium.

As with vasomotor function, genetic background can influence the transcriptional responses to exercise training. Although the overrepresented pathways appeared similar between HIT groups of SJL and NON, the molecular/cellular functions of gene sets were different between two groups (Table 3.6). A gene set differentially expressed in SJL HIT was anticipated to increase ROS production. Additionally, the top disease identified by IPA analysis for the gene set was 'aortic aneurysm' (data not shown, p = 8.65E-03). Therefore, these findings are in line with increased nitrotyrosine level in skeletal muscle (Fig. 3.10) and ultimately, impaired endothelial function observed in SJL HIT. In contrast, a gene cluster differentially expressed in NON HIT was predicted to decrease adhesion and migration of endothelial cells (Table 3.6). The prediction fits with the concept that exercise training is associated with atheroprotective changes in the endothelium including decreased adhesiveness for inflammatory molecules (206). Thus, the predicted molecular/cellular functions for the gene cluster are also in agreement with the higher endothelium-dependent vasorealxation observed in NON HIT than SED.

In addition to the atheroprotective effects of exercise training on the endothelium, prolonged exercise training is associated with vascular remodeling, resulting in larger blood vessels (32, 137, 218, 277). In NON MOD, differentially expressed genes were connected to molecular/cellular functions of increased endothelial cell development and proliferation (Table 3.6). With the notion that outward vascular remodeling of blood vessels in response to exercise training can lead to decreased acute responses to

endothelium-dependent vasodilators and increased blood flow (32, 96, 97), 4-weeks of moderate intensity exercise training in NON might have elicited a transcriptional response leading to vessel remodeling after a more prolonged training protocol.

In summary, a strain survey for endothelial responses to exercise training across 20 different inbred mouse strains revealed that the traditional exercise training had minimal effects on aortic endothelium-dependent vasorelaxation. In four inbred mouse strains chosen based on the strain survey, baseline mitochondria oxidative enzyme activity and nitrotyrosine abundance in skeletal muscle were variable across inbred strains, suggesting an intrinsic influence of genetic background. Most importantly, it was found that exercise training has non-uniform effects on endothelial function and transcriptional activation of endothelial genes depending on the interaction between genetic background and training intensity. These findings indicate that the optimal intensity of exercise training to improve endothelial function is modified by genetic background. Results from skeletal muscle oxidative enzyme activity, abundance of oxidative stress and pathway analysis for gene expression profiles further support the intricate interaction between genetic background and training intensity on responses to exercise training. The present study provides evidence of an interactive effect between genetic background and training intensity on exercise-induced vascular adaptation. Further studies incorporating larger scale expression profiles, for example, RNAseq or microarray, on different genetic backgrounds might be helpful in expanding our knowledge of the mechanism in regulating endothelial responses to exercise training.

4. SUMMARY AND CONCLUSION

4.1. Summary

The main purposes of the present studies were 1) to identify quantitative trait loci (QTL)/candidate genes residing in the QTL responsible for intrinsic endothelial function and 2) to determine the interaction between genetic background and training intensity on the endothelial adaptations to exercise training.

The first series of experiments were conducted to test the hypothesis that intrinsic endothelium-dependent vasorelaxation is largely variable across inbred mouse strains, and the variation is influenced by one or more QTL. To do so, vasoreactivity was assessed in isolated thoracic aortas from young mice (n=6-10) of 27 inbred strains. The major findings of this study can be summarized as follow: 1) A wide range of differences was found for vasoreactivity, excluding SNP Max. In particular for endothelium-dependent vasorelaxation to ACh, there were ~2 and ~18 fold differences between inbred strains having the lowest and the highest ACh Max (%) and in molarity of ACh IC_{50}, respectively. 2) There were moderate, but significant, correlations between ACh and SNP responses, while ACh responses were not correlated with contractile responses. In contrast, there were some significant correlations between SNP and vasocontractile responses. 3) GWAS revealed several significant and multiple suggestive QTL associated with strain-dependent variation in vasoreactivity. GWAS for responses to ACh identified 4 significant QTL on 3 different chromosomes, all of which were

regions of shared synteny for CVD-related traits in rats and/or humans. In addition, 18 suggestive QTL on 14 different chromosomes were identified, containing several putative candidate genes associated with endothelial function and/or identified by human GWAS for CVD traits. Several suggestive QTL (including one significant QTL for KCl EC_{50}) for responses to SNP, PE and KCl were also identified.

In accordance with the hypothesis, intrinsic endothelium-dependent vasorelaxation as well as other vasoreactivity in thoracic aortas were largely variable across inbred mouse strains. These results provided strong evidence that intrinsic endothelial function, and more generally vascular function, is genetically regulated. Moderate genetic correlations between ACh and SNP responses would be expected because both increase the influx of NO into vascular smooth muscle. This is further supported by the finding that three suggestive QTL overlap between ACh Max and SNP IC_{50}. One of the QTL (Chr. 2) also overlaps with PE Max. Thus, these QTL likely contain genetic factors associated with NO-mediated vasomotor tone regulation in smooth muscle. *Pkig* found in the overlapping suggestive QTL (Chr. 2) might be possibly one candidate. Overall results of correlations between vasoreactivity lead me speculate that common genetic factors exist between smooth muscle relaxation and contraction, but not between endothelial function and smooth muscle contraction.

As expected, GWAS identified several significant and suggestive QTL associated with intrinsic endothelial function. These findings demonstrate that endothelial function is influenced by multiple genetic factors. Notably, none of well-characterized genes, e.g. eNOS and SOD-1, were identified in the QTL. This suggests

that the variation in intrinsic endothelial function in young mice can be primarily attributed to undefined genetic factors. Instead, significant QTL contain a few candidate genes, e.g. *Glrx2*, *Fam5c* and *Trpm3*, which are likely to have roles in endothelial function regulation. The identities of these candidate genes as well as several putative candidate genes residing in suggestive QTL are reinforced by previous findings from rat and human QTL/GWAS studies for cardiovascular traits. Further physiological or molecular analyses are recommended to investigate the role of the proposed candidate genes in endothelial function.

Based on the variation in intrinsic endothelial function and known differences in responses to exercise training, the second series of experiments were conducted to test the hypothesis that endothelial adaptations to exercise training are variable across inbred strains of mice and the variable adaptations to exercise training are dependent on training intensity. First, the strain survey for the effect of traditional exercise training on vasoreactivity was conducted in thoracic aortas from 20 inbred mouse strains to characterize the effect of genetic background on endothelial responses to a commonly used training program in mice. Then, four inbred mouse strains were chosen and the effect of training intensity on vasoreactivity was assessed after 4 weeks of moderate intensity continuous exercise training (MOD) and high intensity interval training (HIT). The major findings from this study were as follow: 1) Aortic rings only from 3 inbred mouse strains (NON/LtJ, A/J, CE/J) among 20 inbred strains showed greater responses to ACh after traditional exercise training compared to sedentary mice within the same strain. The majority of aortic responses to SNP, PE and KCl were not markedly changed

in trained mice compared to sedentary mice as well. 2) In four selected inbred mouse strains (129S1, B6, NON, and SJL), aortic responses to ACh after exercise training varied by both inbred strain and training intensity. Neither MOD nor HIT had effects on responses to ACh in 129S1 and B6. In contrast, both NON MOD and HIT had greater responses to ACh than NON SED. Surprisingly, responses to ACh were impaired in SJL HIT compared to SED. 3) Training-induced changes in endothelial gene expression were also different depending on both inbred strain and training intensity. Overall, the number of differentially expressed genes was proportional to the change in endothelial function. The number of genes altered by HIT was greater than MOD and there was little overlap between genes altered by HIT and MOD. Genes differentially expressed in HIT were overrepresented in pathways related to inflammatory responses, while genes differentially expression in NON MOD were enriched for vessel growth-related pathways.

Contrary to the hypothesis, endothelial responses to traditional exercise training were not variable across inbred mouse strains. These were unexpected results. It can be speculated that traditional exercise training intensity was not high enough to stimulate functional changes in endothelium-dependent vasorelaxation, especially in such young mice. This possibility prompted me to investigate the effect of high intensity interval training on endothelial function in selected inbred mouse strains.

Endothelial responses to exercise training were variable across selected inbred mouse strains depending on training intensity. These data indicate that a significant interactive effect exists between mouse strain and training intensity on endothelial

responses to exercise training. Both training intensities improved endothelial function in NON. This would be expected since NON had relatively low intrinsic endothelial function (the lowest ACh Max), thus had much 'room under the ceiling' for improvement. B6 and 129S1 had no changes in endothelial function after exercise training with either training intensity even though these strains showed increased mitochondrial enzyme activity after exercise training. These two strains had relatively moderate to good intrinsic endothelial function, implying that the endothelium in these two strains might be optimal. Thus it is feasible to assume that exercise training cannot augment endothelial function above 'the ceiling' in these two strains. Unexpectedly, impaired endothelial function was observed in SJL HIT. SJL HIT had increased nitrotyrosine level in skeletal muscle, therefore impaired endothelial function in SJL HIT would be associated with systemic increase in oxidative stress.

Variation in training-induced transcriptional activation of endothelial genes among inbred strains and between training intensities indicates the interactive effect between genetic background and training intensity. Given the necessity of system-level understanding for complex traits, differentially expressed genes by exercise training were analyzed by Ingenuity IPA. Overrepresented canonical pathways diverged between MOD (vessel growth) and HIT (inflammation), providing further evidence that there is an intensity-specific pattern of training-induced transcriptional activation in endothelium. The molecular/cellular function for genes altered in SJL HIT is predicted to upregulate ROS production, which in accordance with elevated nitrotyrosine level in skeletal muscle. This further supports our conclusion that impaired endothelial function in SJL

HIT is due to increased oxidative stress. In contrast, molecular/cellular function for genes altered in NON HIT is predicted to downregulate cell adhesion, indicating decreased atherosclerotic lesion formation that would contribute to improved endothelium-dependent relaxation. These overrepresented molecular/cellular functions provide the rationale for the opposing effects of HIT on endothelial function between SJL and NON.

Taken together, although mechanistic structure cannot be firmly drawn, it is important to note that the present findings provide the initial advancement in the large genome scale for elucidating genetic basis for intrinsic endothelial function and its responses to exercise training. The findings from GWAS indicate that there are previously unsuspected genetic factors responsible for intrinsic regulation of endothelial function. Further investigation is required to validate the potential candidate genes identified in the present study. Two types of investigations are highly recommended to refine and validate findings from the present studies: 1) Expression QTL, which compares the single nucleotide polymorphism with the gene expression level (3). This approach would be able to refine the roles of single nucleotide polymorphisms located in non-coding regions as transcriptional regulators. 2) Haplotype analysis for sequence variants around a candidate gene (in linkage disequilibrium block). This could confirm the causative variants of the candidate gene in endothelial function regulation (193). The findings from the exercise study indicate that exercise training has non-uniform effects on endothelial function and transcriptional activation of endothelial genes, depending on the interaction between genetic background and training intensity. These findings

emphasize the necessity to consider individual genetic predisposition and exercise intensity, particularly high intensity exercise, to design a training program for maintaining/improving endothelial health. If the present findings could be confirmed in independent datasets using expanded expression profiling to larger scale analyses and/or different sets of inbred strains, these might allow more comprehensive understanding of the mechanisms for endothelial adaptations to exercise training. The interactive effect of genetic background and exercise intensity on training-induced vascular remodeling might also be useful for finding the mechanism for the lack of training-induced endothelium-dependent relaxation in certain inbred strains.

4.2. Limitations

Some limitations should be considered to interpret findings in this dissertation. The isometric tension measurement in the myograph system used in the present studies allows investigators to examine mechanisms for vasomotor responses in isolated vessels to pharmacological stimuli under controlled conditions, however vasomotor responses to shear-induced forces cannot be assessed in this experimental setup. Thus, mechanosensory mechanisms in endothelial function were not considered in the present studies. In addition, this experimental setup was prepared *in vitro*, thus several *in vivo* factors possibly influencing vasomotor tone were excluded, for example, no innervation from nerve ending and no circulating vasomotor molecules (e.g. adenosine, lactate acid) (43, 113, 154).

Mouse thoracic aortas were utilized to assess vasoreactivity in the present studies. Numerous studies have provided evidence that both basal endothelial function and endothelial responses to exercise training are heterogeneous across the vessel size and location in humans and animals (75, 97, 137, 158, 182, 205, 225, 244). For example, Ferrari and colleagues found considerable differences in gene expression patterns and enriched pathways/biological processes between internal mammary arteries and aortas from coronary artery disease patients (75). Similarly, there were different gene expression profiles and enrichments between mesenteric arteries and aortas from young male rats (225). For the response to exercise training, the magnitude of improvement in endothelium-dependent dilation via exercise training was not correlated between resistance and conduit vessels in human subjects (97). Padilla et al. reported markedly different gene expression profiles activated by exercise training between brachial arteries and internal mammary arteries in young healthy animals (205). These previous data raise the possibility that genetic factors influencing intrinsic endothelial function and its responses to exercise training might be vessel-specific.

Sex differences have been also suggested as a factor influencing vascular function (30, 37, 140, 155, 186, 276). In a large cohort of young subjects, men had markedly lower brachial artery FMD (%) compared to women (140). Femoral arteries from female adult pigs also exhibited greater endothelium-dependent vasorelaxation compared to femoral arteries from male adult pigs (155). This might be attributed to sex hormone effects, particularly estrogen which is known to play a protective role in endothelial function related to vasomotor tone, vascular inflammation and vessel repair

(186). This is further supported by a finding that a rapid decline in FMD occurs at the time of menopause in women (37). After exercise training, adult female animals also showed greater improvements in endothelium-dependent vasorelaxation than male adults (155). Females had higher levels of eNOS and SOD protein expression in the sedentary state and also greater increase in those protein expression after exercise training compared to male (156). These data indicate the potential difference in genetic regulation of intrinsic endothelial function and endothelial responses to exercise training between sexes. Accordingly, the findings from male mice in the present studies would be limited for their generality and applicability to both sexes. Proposed vessel- and sex-specific differences in genetic contribution to endothelial function represent an intriguing area for future research.

4.3. Clinical relevance

Our GWAS revealed several single nucleotide polymorphisms which were significantly associated with variation in intrinsic endothelial function in mice. QTL encompassing these single nucleotide polymorphisms contain a few candidate/putative candidate genes potentially linked to functions related to baseline endothelial regulation. These results provide novel insights into previously unsuspected mechanisms for endothelial function. Elucidation of these mechanisms will have the potential to enhance prediction of endothelial dysfunction and develop into therapeutic targets for CVD associated with endothelial dysfunction. The results of the second study indicate that the effect of exercise training on endothelial function is influenced by the interaction

between genetic background and exercise intensity. These findings suggest new perspectives for the optimization of exercise training to exert beneficial effects on endothelial function and ultimately provide potential to aid the development of individualized exercise training program required to maintain or improve endothelial health.

The present studies were the first to conduct genome-wide exploration for the variation in intrinsic endothelial function in a large cohort of inbred mouse strains and characterize the interaction of genetic regulation and training intensity on endothelial responses to exercise training. Therefore, the present studies represent the first step toward the comprehensive discovery of genetic determinants that regulate intrinsic endothelial function and its responses to exercise training.

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