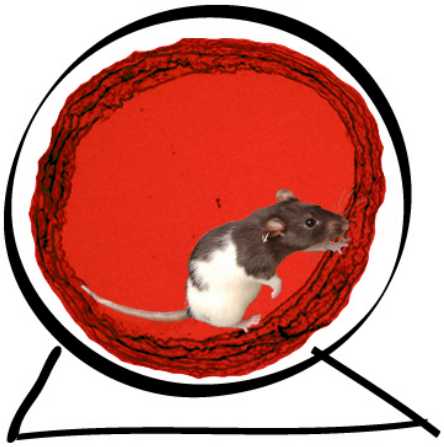
Per Magnus Haram

Genetic vs. Aquired fitness: Metabolic, Vascular and Cardiomyocyte Adaptations



Doctoral thesis
for the degree of philosophiae doctor

Trondheim, June 2006

Norwegian University of Science and Technology
Faculty of Medicine
Department of Circulation and Medical Imaging

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CONTENTS

PREFACE

The work of this thesis has been carried out at the Department of Circulation and Medical Imaging, Faculty of Medicine, Norwegian University of Science and Technology during the years 2002-2006. The working hypothesis of the studies was that diverging aerobic capacity, either inherited or acquired, correlates with metabolic and cardiovascular adaptations both in the sedentary state and in response to exercise.

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DEFINITIONS

Artificial selection: In a genetically isolated population, random mating is prevented and mating is limited to those individuals who exhibit desired characteristics.

Inbreeding: Mating closely related individuals to facilitate the weeding-out of undesired characteristics and the fixation of desired traits.

Maximal oxygen uptake: The highest oxygen uptake the individual can attain during exercise engaging large muscle groups dynamically while breathing air at sea level. It is probably the best indicator of cardiorespiratory performance.

Endothelial function: This includes several functions of the endothelium, in this thesis it is indicated by acetylcholine-induced nitric oxide mediated vasodilation.

Metabolic syndrome: Complex clustering of cardiovascular risk factors defined by the International Diabetes Federation as: Central obesity including any two of the following four factors: a) raised triglyceride levels or specific treatment for this lipid abnormality, b) decreased high-density lipoprotein levels or specific treatment for this lipid abnormality, c) raised blood pressure: systolic Bp ≥ 130 mm Hg or diastolic Bp ≥ 85 mm Hg, or treatment for previously diagnosed hypertension, d) raised fasting plasma glucose > 100 mg/dL (5.6 mmol/L) or previously diagnosed type 2 diabetes.

Detraining: Complete withdrawal of physical activity after a prolonged exercise period.

Fractional shortening: The decrease in cardiomyocyte length from end-diastole to end-systole divided by end-diastolic length; defines the degree of shortening.

Intracellular calcium concentration ($[Ca^{2+}]_i$) transient: The transient increase and decay of $[Ca^{2+}]_I$ during a contraction-relaxation cycle of the cardiomyocyte; denotes the cytosolic Ca^{2+} changes that induce contraction and relaxation.

BACKGROUND

Exercise and health

The human genome was selected through natural selection to maximize fitness in the early ancestral environment, a time in which physical activity was obligatory for survival. Our genome has not changed much the last 100 000 years, and exercise still remains essential for optimal gene expression and avoidance of disease[24,25,30]. Indeed, physical inactivity is now established as an independent risk factor for cardiovascular morbidity and mortality, an effect that is similar to that of high blood pressure, high levels of blood lipids and smoking combined[5]. The human body is therefore not ideally suited for modern Western lifestyle, where inactivity is the norm with a daily energy expenditure corresponding to 38% of what our Paleolithic ancestors had[24,25,30]. An inactive lifestyle will therefore alter gene expression and perturb homeostasis in several organ systems towards an un-physiological range and lead to complex disease scenarios such as the metabolic syndrome.

The metabolic syndrome

In 1979, Kannel and McGee[92] discovered increased incidence of cardiovascular disease in patients with diabetes. Almost a decade later, Reaven[151] described the Metabolic Syndrome consisting of 3 or more of the following criteria; central obesity, atherogenic dyslipidemia, raised blood pressure, insulin resistance or glucose intolerance, prothrombotic state, and proinflammatory state. According to the International Diabetes Foundation (http://www.idf.org) the new consensus worldwide definition of the metabolic syndrome, for a person to be defined as having the metabolic syndrome they must have: central obesity plus any two of the following four factors: raised triglyceride level, reduced HDL cholesterol, raised blood pressure, raised fasting plasma glucose or previously diagnosed type 2 diabetes. The metabolic syndrome is now present in at least 25 % of the US population (American Heart Association). The metabolic syndrome is a multifactorial disease caused by interactions between multiple genetic and environmental factors, and several studies link impaired aerobic metabolism to the pathogenesis of the metabolic syndrome in humans[124,141]. A limitation in studies indicating a cause-effect relationship between the metabolic syndrome and aerobic metabolism in humans is that one cannot exclude the possibility that the observed impairment in metabolism may be caused by other health behaviours not measured. An animal model therefore seems to be the only suitable model to test whether there is a cause-effect

relationship between impaired aerobic metabolism and occurrence of the metabolic syndrome. At the start of the project, it is not known whether animals selected on the basis of low versus high intrinsic exercise performance would also differ in maximal oxygen uptake, mitochondrial oxidative pathways, and cardiovascular risk factors linked to the metabolic syndrome.

Aerobic capacity

Aerobic capacity consists of maximal oxygen uptake (VO_{2max}), anaerobic threshold (Th_{an}) and work economy[217].

Most previous work regards maximal oxygen uptake (VO_{2max}) as the single best indicator of an individual's cardiorespiratory endurance capacity[217]. Although traditionally related to endurance performance such as cross country skiing and running, VO_{2max} has been established as a strong predictor of cardiovascular morbidity and mortality[127]. Improved VO_{2max} can be acquired through endurance training and is associated with salutary adaptations in multiple organ systems. A determination of VO_{2max} offers a precise measure of the capacity to transport and utilize oxygen; that is the functional capacities of the lungs, cardiovascular system, and muscle mitochondria combined. At maximal aerobic exercise, the majority of evidence demonstrate a VO_{2max} that is supply limited[154,155,156,166,167]. This appears to be evident in highly trained athletes[145] and in average fit humans[101]. Consequently cardiac output and more precisely stroke volume, as maximal heart rate is inborn, has a major influence on VO_{2max}[125,166,167,200]. This conclusion is based on the observation that the capacity of skeletal muscle to consume oxygen markedly surpasses the capacity of the heart to supply oxygen. It is estimated that only one third of the muscle mass of man can fully utilize the capacity of the heart[166,167,200]. If a larger muscle mass is intensely engaged in the exercise, sympathetic vasoconstriction occurs in the arterioles of the exercising limbs to avoid a reduction in blood pressure[155,156]. Blood flow in healthy arteries is therefore indirectly restricted by cardiac output, and not by the ability of arteries to dilate. The capacity of the muscle capillary network is never reached at maximum exercise[14,155,156,200], but a denser capillary network exists in endurance athletes. This might prolong the transit time of erythrocytes to allow for increased extraction rates of oxygen and substrate exchange[70]. At the skeletal muscle level, the oxidative capacity of mitochondria could restrict VO_{2max} not only through restrictions in the systemic supply of oxygen, but also by limitations in extraction of oxygen, diffusive oxygen transport from the muscle capillary to the mitochondrial cytochrome. Approximately

98% of the oxygen we metabolize is handled by our mitochondria, and exercise training increases mitochondrial density, size, and enzyme activity[161]. Two important metabolic effects of enhanced mitochondrial enzyme activity include 1) increased capacity to oxidize fat at a higher rate (thus sparing muscle glycogen and blood glucose) and 2) a decreased lactate production during submaximal exercise[98,179,197]. These muscle adaptations are important in explaining the improvement in endurance performance that occurs with training[137] since metabolic adaptations in skeletal muscle are critical for improving submaximal endurance performance. There also exists evidence that untrained humans are demand-limited and improvement in VO_{2max} early in the training period is produced by peripheral factors[76,126,200].

Th_{an} determines the fraction of VO_{2max} that may be sustained for an extended period of time[187,217], and represents the highest intensity during dynamic exercise with large muscle groups, in which production and clearance of lactic acid are approximately the same during a steady rate work condition[135,187,217]. The factors determining Th_{an} are not well known, but muscle fiber type distribution, the potential for fat metabolism, and skeletal muscle lactic dehydrogenase isoenzyme distribution may be important determinants[135,187,217].

Work economy is referred to as the ratio between work intensity and oxygen consumption[36,78,169]. At a given work intensity, oxygen uptake may vary considerably between subjects with similar VO_{2max}. This is evident both in highly trained[36] and in untrained subjects[15]. In elite endurance athletes with a relatively narrow range in VO_{2max}, work economy has been found to differ as much as 20 %[182] and to correlate with performance[36,78]. The causes of intra-individual variations in gross oxygen cost of activity at a standard work intensity are not well understood, but it seems likely that anatomical traits, mechanical skill, neuromuscular skill, and storage of elastic energy are important[78,135].

Endothelial function

Furchgott and Zawadzki[61] discovered the importance of the endothelium, the innermost cell layer lining the cardiovascular system, in the regulation of vascular tone. It has since been acknowledged as an organ with important autocrine and paracrine functions. A large number of vasoconstrictive and vasodilating substances are produced in endothelial cells to act on the underlying vascular smooth muscle cells. Nitric oxide (NO), probably the most important endothelial-derived relaxing factor, is produced by the endothelial isoform of nitric oxide synthase (eNOS). In addition to relaxing vascular smooth muscle, NO counteracts the

formation of atherosclerosis through inhibition of leukocyte adhesion and invasion, smooth muscle cell proliferation, platelet aggregation, and inflammation[43]. Abnormalities in one or more of the pathways that ultimately regulate the availability of NO (See figure 1) may lead to endothelial dysfunction, which is characteristic of cardiovascular disease and is found in patients with coronary risk factors including hypertension, hypercholesterolemia, cigarette smoking, diabetes mellitus, and obesity. Importantly, endothelial dysfunction, as defined by impaired endothelial-dependent vasorelaxation, has been identified as an independent risk factor and a strong prognostic marker of long term cardiovascular morbidity and mortality in latent and manifest cardiovascular disease[56,172]. Endothelial dysfunction plays an important role in the early pathogenesis of atherosclerosis[43] and impaired endothelial function has been observed several years ahead of traditional markers of cardiovascular disease[43,172]. Thus, the preservation of endothelial NO-production and bioavailability should be a major therapeutic goal.

Production of NO

NO is synthesized from l-arginine by eNOS following stimulation by either shear stress or endothelial agonists such as bradykinin or acetylcholine. Agonist occupation leads to increased endothelial [Ca^{2+}] which activates AMP-activated protein kinase (AMPK) and CaM-dependent kinase II (CaMKII) leading to phosphorylation of eNOS at SER-1177. Phosphorylation of the same residue can also occur independently of Ca^{2+} during mechanical stimulation with shear stress as a consequence of the sequential activation of phosphatidylinositol 3-kinase (PI3K)[119] (See figure 1). Furthermore, a complex reaction occurs involving the transfer of electrons from nicotinamide-adenine dinucleotide phosphate (NADPH), via flavins in the carboxy-terminal reductase domain, to the heme in the amino-terminal oxygenase domain, where the substrate l-arginine is oxidised to l-citrulline and NO[8]. Besides eNOS there exist two other NO-synthase isoforms; inducible and neuronal; where eNOS and the latter exist in the endothelium. NOS functions as a dimer consisting of two single monomers, each with a separate C-terminal reductase domain and an N-oxidase domain and a calmodulin site responsible for Ca^{2+}-activation. For appropriate function of the enzyme, the monomers are connected depending upon heme and tetrahydrobiopterin (BH4). High abundance of BH4 and also the eNOS associated protein heat shock protein 90[13,62] (Hsp 90) makes the NOS a pure NO-synthesizer, while decreasing availability of BH4 and Hsp 90 ultimately transforms NOS into a producer of superoxide anions (O_2^-)[178].

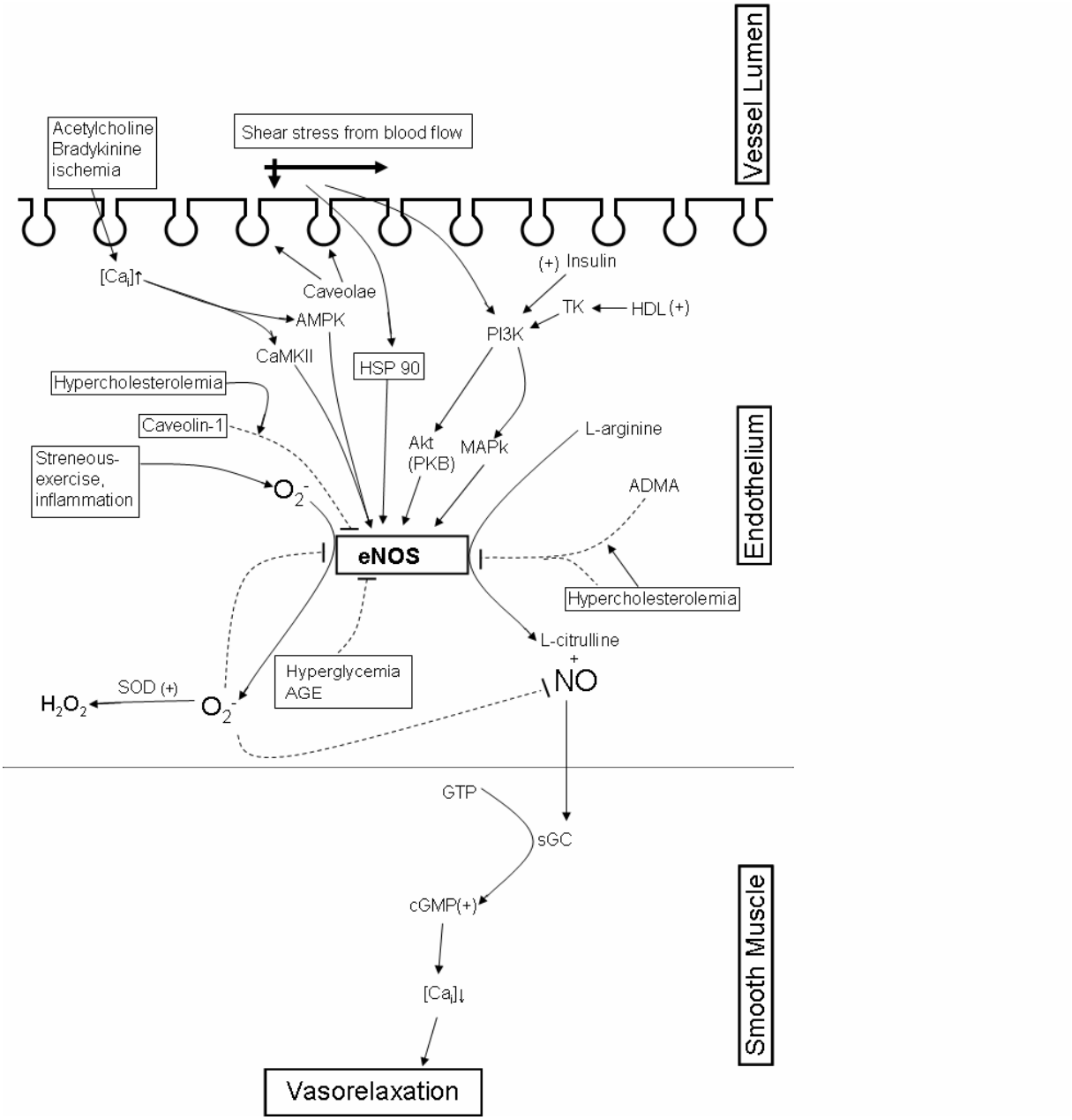

Figure 1: *Some factors influencing the NO-bioavailability in the endothelium, arrows define stimulating pathways, while bars define inhibitory pathways. See text for further details. AMPK, AMP-activated protein kinase; CamKII, Calmodulin-dependent protein kinase II; TK, tyrosine kinase; HDL, high density lipoprotein; PI3K, phosphatidylinositol 3-kinase; ADMA, asymmetric dimethylarginine; SOD, super oxide dismutase;* O_2^-*, superoxide anion; MAPk, mitogen activated protein kinase; eNOS, endothelial nitric oxide synthase; AGE, advanced glycosylation end products; NO, nitric oxide; GTP, guanosine tri-phosphate; sGC, soluble guanosine cyclase; cGMP, cyclic guanylyl mono-phosphate.*

Regulation of eNOS expression

Both shear stress and regular exercise have been shown to upregulate eNOS expression, and a shear stress responsive increase in eNOS-mRNA in bovine aortic endothelial cells has been shown to be dependent upon Ca^{2+} [210] and G-proteins[117]. Other stimuli known to upregulate eNOS are: hypoxia[11], vascular endothelial growth factor[81], HMG-CoA reductase inhibitors[108], and hyperthyroidism[35]. eNOS can also be downregulated by factors such as TNF-α[4], oxidized low density lipoprotein[22], hypothyreosis[35], hypertension[39] and by distortions in lipid-metabolism[160,209]. Furthermore, partial deletion of the eNOS gene increases susceptibility to high-fat diet-mediated arterial hypertension[37], while mice with double knock-out of the eNOS gene produces a phenotype that mimics the metabolic syndrome including insulin resistance, hyperinsulinemia, dyslipidemia, and hypertension[38,53]. Also caloric restriction increased mitochondrial biogenesis in wild type but not eNOS null mice[130]. This suggests that lower abundance of eNOS is a molecular link between cardiovascular and metabolic diseases. The cause-effect relationship in this process is not clear, but it has been suggested that perturbation of lipid metabolism causes early abnormalities in acetylcholine-dependent relaxation and decreased eNOS expression in arteries[160,209].

Oxidative stress and bioavailability of nitric oxide

Endothelial function is dependent upon the balance of oxidant and antioxidant mechanisms. An imbalance in redox state where pro-oxidants overwhelm anti-oxidant capacity results in oxidative stress[33]. Superoxide anions (O_2^-) will then decrease the function of eNOS and reduce the half-life of NO by increasing the production of peroxynitrite from NO and O_2^-. This reaction is associated with pathological conditions; while in normal conditions, O_2^- is quenched by super-oxide dismutase (SOD). Reactive oxygen species (ROS) also regulate vascular function by modulating cell growth, apoptosis, migration, inflammation, secretion, and extracellular matrix protein production[75]. Oxidative stress and associated oxidative damage are mediators of vascular injury and inflammation in many cardiovascular diseases, especially when complicated with hypertension, hyperlipidemia, and diabetes. The major source of oxidative stress in the artery wall is NADPH oxidase. In addition, xanthine oxidase, uncoupled nitric oxide synthase, and mitochondrial leakage of ROS during oxidative reactions can also produce stress. Obesity and the metabolic syndrome is associated with raised oxidative stress, and recent studies have revealed that inflammatory and stress-response genes are among the most abundantly regulated genes in adipose tissue of obese animals[203].

Vessels and shear stress

Blood vessels are different depending upon the task which they perform. From the left ventricle, pressure-compliant large conducting arteries rich in elastin, divide into more muscular arteries capable of controlling blood flow and pressure. The blood flows into the capillaries and from there over in the thin-walled venous system before entering the right atrium. The layer closest to the bloodstream, the endothelium, remains largely unchanged throughout the cardiovascular system. The endothelium is constantly exposed to hemodynamic forces varying in magnitude and direction depending upon the anatomy of the blood vessel and of the viscous drag from the blood flowing through it. Forces acting on an artery due to blood flow can be divided into two principal vectors. One is perpendicular to the wall and the other acts parallel to the wall to create a frictional force which together exert shear stress at the surface of the endothelium[45,46,131]. This is an important physiological stimulator of the endothelium, and it is involved in stimulation of NO-production, vascular remodelling and blood vessel formation[153]. Vascular shear stress increases during exercise and applying shear stress to cultured endothelial cells have been an important technique in the discovery of how exercise-mediated shear stress may affect endothelial cells[45,46,153] .

Sensing of shear stress and intercellular signalling

The mechanotransduction pathways transforming mechanical shear stress into alterations in gene expression are unknown, but many pathways have been proposed[45,46]. A central hypothesis concerns the numerous 50-100 nm invaginations of the endothelial plasma membrane called caveolae. Caveolae consist of the governing protein caveolin-1 together with phospholipids, sphingolipids, and cholesterol, and may be central in regulating the NO-production[176,177]. Proposed functions of caveolae are vesicular transport and contributors to cholesterol homeostasis, but in recent years, the "Caveolae signaling hypothesis"[168,180] stated caveolaes importance in signal transduction, suggesting caveolins serve as oligomeric docking sites for organizing and concentrating signalling molecules within caveolae membranes[150]. This is also true for the endothelial isoform of NO-synthase (eNOS) which is bound to and inhibited by caveolin-1 in the caveolae. Lessons from the caveolin (-/-) mice[52,149] further supports this by exhibiting unstable basal vessel tone and vigorous acetylcholine mediated NO production which indicates a lack of inhibiting caveolin-1. Chronic shear stress stimulates caveolae formation by translocating caveolin-1 from the Golgi-apparatus to the luminal plasma membrane[29] which leads to enhanced sensitivity to shear stress with an increased phosphorylation of eNOS[159]. Shear stress[44,148] is also associated with a rapid upregulation of

eNOS protein and mRNA. The serine/threonine protein kinase, Akt, has been shown to be up-regulated in cultured endothelial cells submitted to shear stress, and it works independently of an increase in Ca^{2+} [51]. Hambrecht[71] found double and triple enhanced expression of eNOS and eNOS phosphorylated at Ser^{1177} (peNOS), respectively, in the left mammary artery of patients with atherosclerotic disease who underwent regular exercise. The total expression of Akt was not upregulated, but the level of phosphorylated Akt (pAkt) was upregulated by 90 %, and there was a close correlation between pAkt and peNOS, accounting for involvement of this pathway in exercise. The output of NO is however only 1/3 when exposed to shear stress versus agonist mediated production, but the shear stress mediated output is maintained for hours versus minutes in the agonist mediated production[57].

Athletes arteries?

It is accepted that there exists a close link between VO_{2max}, regular endurance training, and endothelial function in humans[68,71,72]. It is widely held that the endothelial benefits from exercise are most pronounced in (and perhaps even limited to) subjects with pre-existing endothelial dysfunction[63]. For example, exercise has been shown to improve endothelial function in humans and rats with metabolic syndrome[12,73] and chronic heart failure[83,115]. In contrast, studies in healthy subjects reveal conflicting data with some showing improved[100], unaltered[184] or even depressed[16] endothelial function. Furthermore, it seems that endothelial function is well preserved in young, healthy women and men[163], and that a high aerobic training status does not increase the dilating capacity. Nevertheless, athletes have larger diameter arteries compared to untrained counterparts, and thus have a larger functional capacity (i.e., blood transporting capacity) of their vessels[87,173]. A large resting brachial artery has been shown to be an independent predictor of significant coronary arterial disease[82]. However in athletes with a high cardiac output, shear stress and endothelial function is preserved despite the larger arterial diameter[163]. This may be an analogue to physiological hypertrophy of the athlete's heart with improved function vs. the pathological hypertrophy (i.e. observed in patients with heart failure) with impaired function. Increased arterial diameter on the basis of an exaggerated stimulated production of NO in athletes[163] can suggest that a structural enlargement of the artery has taken place in athletes. The mechanisms responsible for mediating vascular structural enlargement are not fully understood, but there is strong evidence that NO plays an important part and that shear stress is the trigging factor[196].

Cardiac function

Athletes heart

Endurance training is often associated with functional and morphological changes in the heart, such as increased left ventricular chamber size, wall thickness, and mass[138,143]. Furthermore, the athletes heart is associated with increased maximal cardiac output with enhancement in both the diastolic filling and ventricular ejection rate[9,55,186].

Cardiomyocyte dimension and contractile function

Since myocardial tissue from trained humans is not easily available, most data on the cellular level are from experimental models. Several animal models of endurance exercise have been shown to mimic important aspects of human physiology and could help determine the cellular and molecular mechanisms of training induced improvements of cardiac function[96,97,205,206,207]. In a rat model of endurance training, increased dimensions and improved left ventricle contraction and relaxation can be observed in isolated cardiomyocytes. This demonstrates that improved intrinsic (i.e., without influence of the neuro-hormonal system) cardiomyocyte function can contribute to both the systolic and diastolic improvements that occur in the athletes heart.

Training-induced elongation of left ventricular cardiomyocytes occurs in the absence of changes in sarcomere length[123] and the changes in cardiac contractile function induced by endurance training are due in part to cardiomyocyte length-independent changes in contractile function. Several lines of evidence support this notion. Schaible and Scheuer[170,171] demonstrated that treadmill training increased end-diastolic volume, stroke work, ejection fraction, and midwall fractional shortening in the absence of changes in end-diastolic wall stress in perfused working rat hearts. Furthermore, isometric force development by rat left ventricular papillary muscle maintained at optimal length is increased by endurance training[122,192,194]. Recently Diffee and Chung[48] showed that training increased the velocity of loaded shortening and increased peak power output in the single permeabilized cardiomyocyte preparation. At slow stimulation frequencies (0.067-0.2 Hz) and low temperatures (23-29° C) there is little evidence of training-induced improvement in the shortening characteristics of cardiomyocytes[111,123]. However, training-induced adaptations, such as increased degree of fractional shortening and reduced re-lengthening time, become more evident as both the stimulation frequency and temperatures approach in vivo conditions[205,214,215]. For the rat this is 300 to 600 beats per minute at 37° C. There seems to be a progressive increase in

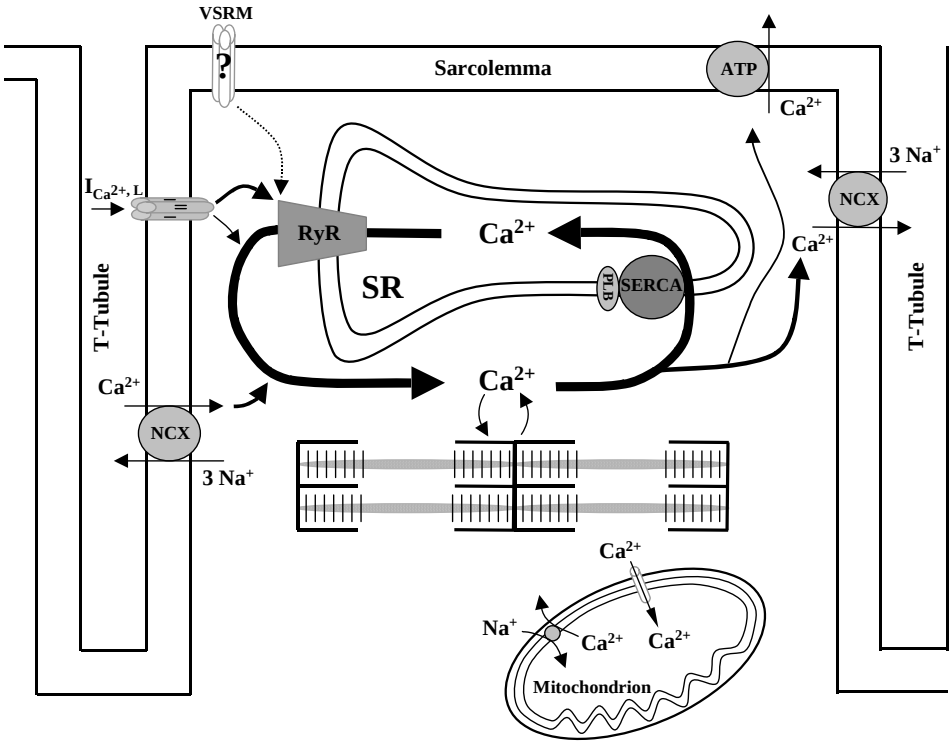

Figure 2. *The main mechanisms that contribute to the excitation-contraction coupling and removal of Ca^{2+} from the cytosol after contraction. VSRM, voltage sensitive release mechanism; ATP, adenosine triphosphate; NCX, sodium-Ca^{2+} exchanger; SERCA, sarcoplasmatic reticulum Ca^{2+} ATPase; $I_{Ca^{2+},L}$, inward Ca^{2+} flux via L-type channels; PLB, phospholamban; RyR, ryanodine receptor. For details, see text. Modified from Bers*[18].

cardiomyocyte contractility in response to regular exercise training until a plateau of training effects has been reached. This coincides with the maximal increase in VO_{2max} and cardiomyocyte hypertrophy[205,206].

Intracellular calcium transients

In cardiac muscle, the force of contraction depends on the peak intracellular calcium (Ca^{2+}) concentration during systole, the sarcomere length, and the responsiveness of the myofilaments to Ca^{2+} [18]. Impairment of Ca^{2+} handling is a major cause of both contractile dysfunction and arrhythmias in pathophysiological conditions[21,144]. A brief increase in cytoplasmic Ca^{2+} concentration allows Ca^{2+} to bind to the myofilament protein troponin C, which activates the myofilaments. This is often called the Ca^{2+} transient and this transduces the chemical signal and energy (ATP) into cardiomyocyte shortening in a Ca^{2+}-dependent manner. During the action potential Ca^{2+} ions enter the cells mainly via voltage-activated Ca^{2+}

channels (dihydropyridine receptors or L-type Ca^{2+} channels) as an inward Ca^{2+} current (i_{Ca2+}).

L-type Ca^{2+} channels are located primarily at sarcolemmal-sarcoplasmatic reticulum (SR) junctions where the SR Ca^{2+} release channels (the ryanodine receptors) exist. In addition, the sodium (Na^{+}) - Ca^{2+} exchanger contributes to Ca^{2+} influx and efflux with a stoichiometry of three Na^{+} to one Ca^{2+} that produce an ionic current either inward (forward mode: during high intracellular Ca^{2+} concentrations) or outward (reverse mode: during positive membrane potentials and high intracellular Na^{+}). The Ca^{2+} entering the cardiomyocyte from the outside contributes directly only to a minor degree to myofilament activation, and its main effect is to stimulate Ca^{2+} release from the intracellular pool of Ca^{2+}: the SR. This is normally termed Ca^{2+} -induced Ca^{2+} release. For relaxation and filling of the heart to occur, the intracellular Ca^{2+} concentration must decline. This requires Ca^{2+} transport out of the cytosol by four pathways involving SR Ca^{2+}-ATPase (SERCA2), sarcolemmal Na^{+}-Ca^{2+} exchange, sarcolemmal Ca^{2+}-ATPase, and mitochondrial Ca^{2+} uniport[18]. The SERCA2 and Na^{+}-Ca^{2+} exchange are most important quantitatively. In rat ventricular cardiomyocytes, the SERCA2 removes about 92% of the activator Ca^{2+} from the cytosol, whereas the Na^{+}-Ca^{2+} exchange removes 7%, with only about 1% each for the sarcolemmal Ca^{2+}-ATPase and mitochondrial Ca^{2+} uniporter. In heart failure the expression of SERCA2 is normally reduced and Na^{+}-Ca^{2+} exchange increased, and both changes tend to reduce the Ca^{2+} content in SR, limiting SR Ca^{2+} release, which may be a central cause of systolic deficit in heart failure[18,144].

Cardiomyocyte shortening in healthy endurance-trained rats is associated with lower peak systolic and diastolic intracellular Ca^{2+} [123,,206,207] and reduced time for the Ca^{2+} decay from systole[123,206,207]. Gene analysis demonstrates a marked up-regulation of SERCA2 and Na^{+}-Ca^{2+} exchanger in trained hearts[188,189,193,206,207]. Chronically elevated Na^{+}-Ca^{2+} exchanger levels are known to reduce systolic Ca^{2+} [190] and may contribute to the reduced peak systolic Ca^{2+} observed in cardiomyocytes from endurance-trained rats. Furthermore, increased Ca^{2+} uptake capacity of the SR due to increased SERCA2 expression could account for the increased rate of decay of the Ca^{2+} transient observed after regular exercise training[206].

Myofilament calcium sensitivity

An additional mechanism for the increased contractile force in the cardiomyocyte is that exercise training may result in an increase in the sensitivity of the myofilaments to activation

by Ca^{2+}. An increase in Ca^{2+} sensitivity would result in a greater level of isometric tension generation at the same intracellular Ca^{2+} level. In healthy rats, treadmill running induces an increased cardiomyocyte sensitivity to Ca^{2+}, both in intact cardiomyocytes[123,207], and in permeabilized cardiomyocytes[48,50,207], with more pronounced changes in endocardial compared to epicardial cardimyocytes[49]. There are also indications that permeabilized cardiomyocytes from trained hearts are less affected by low pH at constant Ca^{2+} than sedentary counterparts[206,207]. As previously reported[2], low pH decreases and alkaline pH increases myofilament shortening in cardiomyocytes from sedentary and trained cardiomyocytes. In an analogous way to intracellular Ca^{2+}, this indicates that a component of the enhanced cardiomyocyte contractility could be attributed to the more alkaline intracellular pH in the trained cardiomyocytes at high stimulus frequencies.

AIMS OF THE STUDY

1. To determine
    a) whether rats selected on the basis of low versus high intrinsic exercise performance also differ in maximal oxygen uptake, mitochondrial oxidative pathways, and cardiovascular risk factors linked to the metabolic syndrome

   To compare
    b) the cardiomyocyte and endothelial function in rats with intrinsic high- and low aerobic capacity

2. To determine whether endurance training can ameliorate the cardiovascular risk profile in rats with inborn metabolic syndrome

3. To determine
    a) the time-dependent adaptation and decay of maximal oxygen uptake, cardiomyocyte contractility and endothelial function in response to training and de-training

    b) in detail the time-dependent increase and decay of endothelial function following a single bout of exercise

    c) in-depth analysis of the time course of endothelial function following cessation of an extended exercise period

METHODOLOGICAL CONSIDERATIONS

Animal models

To study cellular and molecular adaptations to exercise we used rodent models as delineated in each paper; all studies were approved by the Norwegian Council for Animal Research and conformed to international guidelines for animal research. For the two first papers (I and II), we used rats selectively bred for high or low aerobic capacity, and they were subsequently termed high capacity or low capacity runners, i.e. HCR and LCR, respectively. It has been shown that aerobic phenotype is a complement of genes that determine intrinsic exercise capacity[27] and an additional set of genes that dictate the adaptational response to exercise[26,28]. Based on this theoretical background, artificial selection for high or low intrinsic aerobic treadmill-running capacity started in 1996. To provide enough variance for intrinsic aerobic capacity, the genetically heterogeneous N:NIH stock of rats was used as the founder population (n = 168)[102]. Selection for low and high capacity was based upon distance run to exhaustion on a motorized treadmill using a velocity-ramped running protocol. The 13 lowest and 13 highest capacity rats of each sex were selected from the founder population and randomly paired for mating. For subsequent generations, one female and one male offspring were selected from each family and became parents for the next generation. At each generation, within-family selection was practiced using 13 families for both the low and high lines. A rotational breeding paradigm maintained the coefficient of inbreeding at less than 1% per generation[102]. After 11 generations, HCR and LCR differed by 347 % in distance to exhaustion, and after 15 generations by 500%. For the two last papers (paper III and IV), we used female Sprague-Dawley rats, which is well characterized from previous studies of similar nature in our laboratory[96,97,116,205,206,207].

Exercise training and testing of maximal oxygen uptake

Based upon experience in our laboratory, as well as clinical trials, it appears evident that cardiovascular adaptations rely on the exercise intensity during long-term regular training programs. A high aerobic intensity is required for optimal outcome of the training program, and a key feature to improve VO_{2max} is to improve the cardiovascular capacity to supply oxygen and metabolites to working skeletal muscles[167]. After it was demonstrated that stroke volume in well-trained athletes increases continuously up to maximal levels at VO_{2max}, the importance of high exercise intensity became evident[89,216]. Indeed, recent clinical trials show that high aerobic intensity is necessary and more beneficial than low-to-moderate intensity to

gain full effect of a training program even in untrained subjects[1,112,162]. Thus, to maximize VO_{2max}, we chose the custom-made treadmill running model as our preferred model of exercise[205]. All exercise sessions throughout the investigations were carried out at 85-90% of VO_{2max}, interspersed by short periods of milder intensity. Training protocols are detailed in each paper. Moreover, uphill treadmill running is a full-body exercise that taxes the cardiovascular system and oxygen uptake system substantially, while concordantly reducing the running speed, which otherwise would have the potential to restrict the animal from reaching satisfactory exercise-intensity during each exercise-bout and VO_{2max} during the tests. The physiological adaptations to this exercise model largely mimic those found in humans, i.e. physiological hypertrophy (of the athlete's heart) and improved function in the heart[9,55,138,143,186], as well as increased VO_{2max}[163]. To maintain the desired relative exercise intensity, VO_{2max} was measured at the start of every training week, and running speed was adjusted accordingly. Thus, a close control of training intensity was achieved. Running economy, i.e. oxygen uptake at a given submaximal running velocity was measured after the warm-up, but before testing VO_{2max}, to avoid accumulation of excess lactic acid. The test protocols are detailed in a paper from our laboratory[205].

Ex-vivo endothelial experiments

In intact animals, many external factors that we are not able to control affect the homeostasis of the vascular system. Hormonal and nervous influence, the rate of flow through the vessel, and blood pressure are examples of this. Therefore, to assess the intrinsic properties of the vascular system, we used an isolated organ bath model. This is a standard method used to study local pharmacological mechanisms and signaling pathways in isolated vessels and allows the exclusion of any influence from higher regulatory systems. We investigated the mechanical properties related to increase/decrease of vessel diameter in segments 2-4 mm long, by mounting the segments onto two L-shaped stainless steel holders. Segments were unavoidably stretched into a non-circular cross-section whose morphology is non-existent in vivo. The holders were connected to a force-displacement transducer and during experimental procedures the vessel diameter was held constant, while the active force was measured isometrically. Optimal resting tension had been determined in preliminary experiments in our laboratory. More detailed procedures concerning organ bath experiments are detailed specifically in each paper.

Cardiomyocytes in experimental studies

The use of isolated cells has been established in many lines of basic cardiological research. In electrophysiology, ionic currents can be characterized more accurately than in intact tissue where extracellular resistance pathways can complicate measurements. The transport of metabolites across the sarcolemma can be studied independently of the influence of other types of cells and transport barriers. However, most reports about metabolism deal with quiescent cells, which obviously have a very low metabolic rate, provided they are intact, and their oxidative phosphorylation is not uncoupled. Thus, their application as a model of a working heart appears to be restricted. However, using electrical stimulation, the metabolic activity of the cells can be gradually enhanced up to those values observed in beating hearts. In this case, the measurement of mechanical parameters during the response of myocytes to the electrical stimulation is of interest. The advantages of using isolated living cells from heart tissue instead of whole tissue is that the cells are fully differentiated and morphologically similar to cells in intact heart but lack interstitial tissue and other cell types which can complicate measurements in intact tissues[41]. Isolated cardiomyocytes can be studied in a well-defined environment without interaction of unwanted neurohormonal factors seen in the intact heart. Using isolated cells also has the advantage that experiments can often be performed with several parallel measurements from the same animal, which is more effective and reduces the number of animals that have to be sacrificed. This model also has some disadvantages. The cells are separated from contact with other cells and the intercellular matrix, and they are quiescent, as there is no contact with any pacemaker tissue. The cells could also be modified during the cell isolation procedure, which may affect the physiological reliability of the isolated cell model. In order to reduce this problem we always simultaneously isolated cells from trained and untrained groups each day.

Collection of blood pressure data

It is accepted that endothelial dysfunction is associated with hypertension. Both acute and chronic exercise in hypertensive individuals is known to reduce blood pressure by 5-7 mm Hg[74]. In paper IV, we discovered that the acute effects of one bout of exercise upon the endothelium was evident 24 hours post-exercise, but non-significant after 48 hours. To avoid this effect of acute exercise upon the endothelium and possibly also on the blood pressure, blood pressures were measured 48 hours after the last exercise session in the last week of the training period in paper II. We used a non-invasive tail-cuff monitor to measure blood

pressure in paper II. In paper I, blood-pressure was measured invasively in the abdominal aorta from chronically implanted transducers.

Anesthesia

The normal routine for sacrificing animals was diethyl ether anesthesia. It provides a quick state of comatose, but with a stable, maintained cardiac function. Moreover, it removes the stress associated with subcutaneous or intraperitoneal injections of anesthesia.

Electron microscopy and detection of caveolae

Electron microscopy is a reliable and common used method used for the detection of caveolae. Glutaraldehyde fixation was used to optimize the ultrastructure of the sections, caveolin-1 staining was not performed, something which would confirm the presence of caveolae. Instead, distinct flask-shaped vesicles (50-100 nm in diameter) were counted as caveolae, which also has been done by others[174].

Choice of arteries

In paper I and III, we used the common carotid artery, while in paper II and IV we used the abdominal aorta inferior to the branching of the renal arteries. Both vessels were used because of their clinical relevance in the systemic circulation and their susceptibility to atherosclerosis. For the further investigations we have planned in our lab, more organ specific vessels like the popliteal artery supplying the soleus muscle, and the coronary arteries supplying the heart will be used as the current equipment did not support vessels of this size.

Allometric Scaling

Since all of the studies involved a long-term follow-up, it had to be considered that body mass might influence the results. Thus, allometric dimensional scaling was undertaken to appropriately normalize VO_{2max}, and cardiac and skeletal muscle weights to body size. Traditionally, one usually relates data to blank body mass; however, it is now well established that this may be misleading[42] and underestimates VO_{2max} in heavier subjects[183]. We scaled VO_{2max} to body mass raised to the power of 0.75, which is in line with empirical studies.

Gene Expression

Gene expression may be measured at the level of specific mRNA or protein. Depending on the gene and stimuli studied, mRNA and protein might be differentially or similarly regulated. Changes in mRNA expression are detected more proximally after inducing the stimuli and

probably reflect the response to stimuli more directly than protein expression. Presumably the amount of protein is more representative of functional effects of gene expression.

Quantification of mRNA

De novo synthesis of proteins starts with the transcription of the DNA gene to mRNA. The information from mRNA is then translated into protein by the ribosomal machinery in the cytoplasm. In general, the number of mRNA copies correlates with the amount of translated protein. The quantification of mRNA is thus an indirect measure of de novo protein synthesis in the tissue. Amplification of mRNA molecules to study gene expression was achieved by a method that combines two sequential enzymatic steps: the synthesis of DNA from the RNA template by reverse transcriptase followed by quantitative PCR using a heat stabile DNA polymerase.

Immunoblotting

Blotting analysis provides a means to identify a molecular species by size without having to physically isolate it. The principle of the technique is simple: The mixture of molecules to be analyzed is subjected to gel electrophoresis, which separates different species of molecules by their size, and to some extent, their electrical charge. The gel matrix routinely used in blotting is formed in a slab, with wells at one end, into which the molecular samples are loaded. The gel is submerged in a buffer and subjected to an electrical current. The molecules migrate across the gel in the electrical field. Since both DNA and RNA carry a net negative charge, nucleic acid samples migrate toward the positive pole of the electrical field. The size limitations of the matrix hinder larger molecules that eventually segregate according to size. Once the electrophoretic separation is complete, the gel is removed from the buffer and nylon filter is placed over it; dry absorbent material is placed over the nylon. With a weight placed on the top, the buffer in the gel is blotted up into the absorbent material, carrying with it the separated molecules, which come to rest on the filter. The filter is then treated to fix the molecules permanently on its surface, producing a mirror image of the original configuration of molecules in the gel. The filter is subjected to probing with a tagged molecule that recognizes the particular molecule of interest. In blotting analysis of proteins (Western Blots) as used in paper I and II, the probe consists of a tagged antibody that recognizes the desired protein. Once the probing process is complete, the nylon filter is washed to remove excess probe and is analyzed, usually by autoradiography. The molecule identified in this way is

visualized as a band, reflecting the shape of the well in the lane where it was originally loaded.

Statistical procedures

As each study operates with a limited number of animals per group, assuming distributional assumptions were not reasonable. Thus, data were analyzed with non-parametric procedures and complemented with one-way and repeated measures ANOVA where appropriate, as outlined in each paper. We investigated which factors best corresponded to integrative adaptations, in our case VO_{2max} and endothelial function, as this could ultimately indicate which factor targets govern the overall clinical outcome. Such attempts were performed by simple and multiple linear regression analyses, which analyze how one variable is influenced by several others working in concert, as described in the respective papers. Although this approach singled out several features, one should not discard those that did not reach statistical significance, as they likely still are important for the biology. The backward stepwise model was chosen to include all independent variables and then remove insignificant ones one at a time until a final model with only significant cellular contributors was achieved. However, no differences occurred when using the forward stepwise model.

RESULTS AND DISCUSSION

This thesis demonstrates that the level of VO_{2max}, whether inherited or acquired, is closely related to cellular structure and function in the cardiovascular system. Furthermore, it documents that endurance training improves cardiovascular health even in individuals with genetically derived metabolic syndrome.

Inherited maximal oxygen uptake and cardiovascular risk profile

A specific aim of the present study was to determine whether rats selected on the basis of low versus high intrinsic exercise performance also differed in maximal oxygen uptake, mitochondrial oxidative pathways, and cardiovascular risk factors linked to the metabolic syndrome. After eleven generations of selective breeding based upon aerobic treadmill running, we obtained contrasting rat lines of Low Capacity Runners (LCR) and High Capacity Runners (HCR). HCR were superior to the LCR for distance run to exhaustion (347%) and VO_{2max} (60%). LCR demonstrated a cluster of risk factors for cardiovascular disease; higher body mass, visceral adiposity, blood pressure, insulin, glucose, free fatty acids, and triglycerides. HCR were higher for economy of running, five measures of heart function, adaptation to exercise, and nitric oxide-induced vascular dilation. The low aerobic capacity in LCR was associated with decreased amounts of transcription factors required for mitochondrial biogenesis and in the amounts of oxidative enzymes in skeletal muscle. Impairment of mitochondrial function may link reduced fitness to cardiovascular and metabolic disease. Although several lines of evidence have demonstrated strong associations between physical fitness and major cardiovascular risk factors[127], our experiments clearly indicate that low aerobic capacity constitutes a physiological basis which predisposes subjects to clinical manifestations of disease such as the metabolic syndrome (Paper I).

Intrinsic maximal oxygen uptake

A central hypothesis of the present work was that diverging aerobic capacity represents a continuum between health and disease. Untrained female LCR rats of generation 11 had VO_{2max} levels of approximately 45 mL $\cdot$ kg$^{-0.75}$ $\cdot$ min^{-1} (Paper I and II) similar to that observed in rats with post-infarction heart failure[206]. Furthermore, their HCR counterparts had a supra-normal VO_{2max} of ~ 70 mL $\cdot$ kg$^{-0.75}$ $\cdot$ min^{-1} whilst VO_{2max} of normal Sprague Dawley rats was ~ 60 mL $\cdot$ kg$^{-0.75}$ $\cdot$ min^{-1} (paper III and IV). Although LCR males and females weighed 39 % and 24 % more than HCR males and females, respectively, multiple regression analysis

revealed that body weight did not account for more than 7% and 14-20 % of the variations in distance run in females and males (paper I).

Previous work in HCR/LCR rats, at generation 7, showed a 12 % ($p < 0.05$) difference in VO_{2max} between the two strains. Although a significantly smaller stroke volume was found in the LCR at hypoxic, but not normoxic conditions, the major determinant of endurance capacity was found to be a higher capacity of oxygen transfer at the tissue level[79] in line with increased capillary density, citrate synthase, and beta-hydroxyacyl-CoA dehydrogenase in skeletal muscle of HCR. These data suggest that most of the genetic adaptations for improved oxygen utilization in HCR are due to "peripheral factors" in the skeletal muscle and not in differences in heart or lung function[85]. These findings are consistent with increased proteins important for mitochondrial function in soleus muscle of HCR (paper I and II) and the fact that VO_{2max} in untrained individuals is mainly limited by "peripheral factors", whereas in trained individuals, there is a supply limitation of oxygen from the heart[166]. However, using HCR/LCR rats from generation 11 (paper I), we also found substantial differences in cardiomyocyte morphology, contractility, and Ca^{2+}-handling, as well as differences in endothelial function between HCR and LCR, which are all major determinants of cardiovascular health and VO_{2max}. Reduced cardiac and endothelial function might also be responsible for the reduced VO_{2max} seen in LCR.

Exercise-induced improvements in VO_{2max}

A specific aim of the present thesis was to test whether genetically derived metabolic syndrome and low VO_{2max} could be ameliorated. We subjected HCR/LCR rats to a training regimen that has been shown to improve VO_{2max} by 37-55% in normal rats (paper III and IV and [205,207]) and 38% in rats with post-infarction heart failure[206]. In response to endurance training, VO_{2max} increased by 43 % and 44 % in HCR and by 38 % and 46 % in LCR in paper I and II, respectively. Importantly, in LCR, endurance training improved the mitochondrial biogenesis, indicated by a 3-fold increase of PGC-1α in the soleus muscle (paper II) along with salutary changes in cardiomyocyte morphology and function, as well as in endothelial function (discussed below). In contrast to most studies[58,65,170,171] we found that exercise training induced a substantial increase in VO_{2max}. This likely results from the high aerobic intensity of the training regimen where VO_{2max} increased on average 10 % per week until it levelled off at weeks 5-6. Differences in training response reported in the literature are probably due to different training regimens used and/or insufficient control of relative exercise intensity. The load required to produce a training effect has to increase as the

performance improves in the course of training[217]. The training load should, therefore, be set relative to the level of fitness of the individual. Christensen[32] demonstrated, in humans, the need for a gradual increase in training load with improved performance, in the case of the effect on heart rate, as early as in 1931. He observed that regular endurance training at a given exercise rate gradually lowered the heart rate and that after a period of training at a higher load, a standard submaximal work load could then be performed with even lower heart rate. The following general principle of training is apparent in a number of parameters, among them VO_{2max}: After adaptation to a given work load is reached, the absolute exercise intensity required to achieve further improvement, has to be increased[217]. A similar training regimen as used in the present thesis has been applied to patients with established cardiovascular disease[162,208] and in patients with metabolic syndrome[195]. Rognmo et al[162] determined the effect of moderate- and high-intensity aerobic interval training in patients with coronary artery disease (CAD) upon peak oxygen uptake, where they equated training volume so that only exercise-intensity differed between the exercise-groups (i.e. the two groups had similar energy expenditure at each exercise session). They found that high intensity interval training for CAD patients was twice as effective in improving VO_{2max} as compared to the CAD patients that trained with moderate intensity. Similar results were found in patients with post-infarction heart failure exercising with intervals at 90-95% of their peak heart rate[208] as well as in patients with the metabolic syndrome[195]. Thus, it seems like this type of interval training is also highly effective for improving VO_{2max} in humans with cardiovascular disease. Interestingly, the level of VO_{2max} rapidly decreased when rats stopped the exercise program, losing half of its exercise-induced increase in VO_{2max} in 2 weeks. This indicates that the substantial improvements in VO_{2max} in rats over several weeks are quickly lost when subjected to an inactive life-style. The number of exercise sessions necessary to maintain VO_{2max} levels is uncertain, but cutting down from 6 to 2 sessions per week is not sufficient to maintain VO_{2max}[80]. Future studies should determine the amount and intensity required to maintain the gain in VO_{2max} achieved after high intensity interval training.

Plasma metabolites, adipokines, and insulin action

In paper I, we found that male LCR rats were insulin resistant compared to the HCR rats, demonstrated by a 131 % ($p < 0.002$) higher level of insulin and 20 % ($p < 0.0007$) higher fasting glucose levels. C-peptide levels were normal in LCR rats, indicating that insulin secretion was preserved. The C-peptide/insulin ratio, however, was reduced in the LCR rats, indicating decreased insulin clearance. Furthermore, consistent with the notion that female

rats are not as likely to develop diabetes as their male counterparts[113], fasting blood glucose levels were similar in untrained female HCR and LCR (paper II) and were not changed by exercise. However, oral glucose challenge increased blood glucose level by 17% ($p < 0.05$) in untrained, but not trained LCR, suggesting glucose intolerance in sedentary LCR females.

Insulin action was reduced in female LCR liver and adipose tissue but not in soleus muscle, as assessed by the ability of insulin to induce tyrosine phosphorylation (pTyr) in the insulin receptor's β-subunit (IR_β) (paper II). Endurance training increased, but did not completely restore insulin receptor phosphorylation and signalling in the liver and fat of LCR, suggesting persistent insulin resistance in these tissues. This was supported by persistently elevated levels of fatty acid synthase (FAS) in liver and adipose tissue, and of fatty acid transport protein (FATP-1) in adipose tissue, as insulin promotes the storage of substrates in adipose tissue, liver, and muscle by stimulating lipogenesis, protein, and glycogen synthesis and inhibiting lipolysis, glycogenolysis, and protein breakdown[165]. The slight increase in insulin action was associated with a modest reduction in the plasma triglyceride level of LCR rats, which could be related to the insignificant effect of exercise on hepatic carcinoembryonic antigen-related cell adhesion molecule (CEACAM1) levels[40,128]. In contrast, endurance training markedly reduced plasma FFAs in LCR by 63 % and fully restored FFA levels to that of sedentary HCR. Likewise, exercise also normalized visceral adiposity in LCR to HCR levels, unlike in sedentary LCR which have a 4-fold ($p < 0.01$) increase in visceral adiposity compared to HCR. This is consistent with the decreased FFA supply from the less developed adipose tissue and normal FFA uptake into soleus muscle, as indicated by intact insulin receptor phosphorylation and normal FATP-1 level in this tissue.

Visceral adiposity also contributes to the pathogenesis of the metabolic syndrome via increased production and secretion of adipokines and inflammatory factors[175], such as leptin, TNF-α, fatty acids, adiponectins, and angiotensinogen. In paper II, we investigated the level of TNF-α, amongst other effects which will be discussed later, increased TNF-α has been found to have adverse effect on insulin action[84]. The adipose tissue exhibited higher mRNA levels of TNF-α (by ~ 4- to 5-fold) in sedentary LCR than found in HCR. Whereas endurance training reduced TNF-α mRNA in HCR by ~3-fold, it did not significantly reduce TNF-α mRNA levels in LCR. Because of the negative effect of TNF-α on insulin action, it is possible that this contributes to sustained insulin resistance in the adipose tissue in addition to endothelial dysfunction in the LCR.

Endothelial Function

Functional adaptations

Individuals with endothelial dysfunction have an impaired ability to carry out maximal exercise, in that VO_{2max} is reduced by at least 20% compared with that in control subjects of similar age and physical activity level[133,152]. In contrast, highly trained endurance athletes may have VO_{2max} and cardiac output more than 80 mL · kg · min^{-1} and 40 L · min^{-1}, respectively, and this challenges the arteries transporting the blood to the active skeletal muscles[217]. These observations suggest that arterial dimension and endothelial function may play an important role in determining an individual's exercise capacity.

Endothelial function was increased by daily exercise in close concert with improvements in cardiac function and VO_{2max} both in HCR/LCR rats and in normal Sprague Dawley rats (papers I-IV). The regular increase in shear stress exerted by increased cardiac output during each exercise bout is thought to be the main mediator of improved endothelial function[63]. Interestingly, we showed that a single bout of exercise was followed by a brief period of reduced endothelial function (figure 3, panel A). Improvement was present, however, 12-24 hours after exertion, followed by a rapid decrease reaching baseline values 48-hours post-exercise. The reduced endothelial function following one bout of exercise was prevented by incubating the vessels with the superoxide scavenger superoxide dismutase (SOD), suggesting that oxidative radicals produced during strenuous exercise decrease the half life of NO and therefore the bioavailability of NO immediately after exercise. Chronic exercise induced a more pronounced improvement in endothelial function, which might be due to a stronger and more efficient eNOS-NO-cGMP pathway, as discussed later (figure 1), endothelial function was reduced to baseline levels by a week of inactivity (figure 3, panel B) (paper IV). Although detraining for one month has been shown to decrease endothelial function in humans with recent myocardial infarctions[199], detraining athletes for one week had no effect upon endothelial function[64].

Exercise-induced improvement in endothelial function is mainly due to increased NO-availability[47,71,72]. We confirmed this in trained healthy rats and in rats diverging in inherited VO_{2max}, by showing that the exercise-induced improvement in acetylcholine-mediated vasodilation was inhibited by L-NAME, which is in line with previous work[71,72,110,209]. In the carotid artery segments used in paper I and III, we saw an increase in the magnitude of relaxation

in the exercised rats and in the HCR vs. LCR rats, respectively, in line with other studies[12]. This was, however, not evident in the abdominal aorta of the corresponding rats in paper II and IV, and contrasts with previous studies[47]. These discrepancies may be due to differences of eNOS expression in different parts of the arterial tree[110,111]. Furthermore, the response to exogenous NO induced similar vasorelaxations and maximal dilations in vessels from all groups in the current thesis, in line with most studies of healthy subjects as well as in patients with cardiovascular disease[41,71,72] indicating intact sensitivity of smooth muscle to NO. However, increased sensitivity of smooth muscle to NO has been reported to be enhanced in coronary arteries of ultradistance runners[77] and in rats exercised for 22 weeks[12]. The reason for these differences may be due to different exercise regimens, as an increase in vascular smooth muscle sensitivity has only been reported after prolonged exercise periods[12,77].

Exercise intensity and endothelial function

It has been suggested that it is necessary to maintain a high exercise-intensity in order to enhance endothelial function in healthy humans. Whereas, in persons with endothelial dysfunction, a lower intensity exercise regime may be satisfactory[63]. In the present thesis, we used a relatively high aerobic exercise-intensity, which has been shown to have detrimental effect upon endothelial function in some studies[67] but positive effects in others[71,72,97]. Paper IV indicates that this may be a matter of when the measurements are being done in relation to the last exercise bout. We observed a biphasic response in endothelial function after both acute and chronic exercise. Interestingly, we have recently shown in humans with the metabolic syndrome that a single bout of high intensity interval training, as used in paper IV, but not moderate-intensity exercise, induces improvement in flow-mediated endothelial function that lasts up to a week[195] (figure 3, panel C). The fact that a single bout of exercise is able to initiate a substantially improved endothelium-dependent vasodilatation may change the way we look upon exercise as a tool in prevention and rehabilitation of cardiovascular disease. The knowledge that the benefits of exercise start after one bout of exercise can be very motivating for individuals starting a rehabilitation program and also for the physicians recommending it. Furthermore, the data suggest that even in highly trained individuals it is not possible to "store" exercise-induced improvements in endothelial function for a long period of time, as a week of inactivity is enough to abolish the effect of 6 weeks of endurance training. Thus, regular exercise is necessary for long term preservation of endothelial function. Future studies should determine the optimal intensity and frequency necessary for

improved endothelial function, both in rats for in-depth study of the cellular and molecular mechanisms and in humans for clinical relevance.

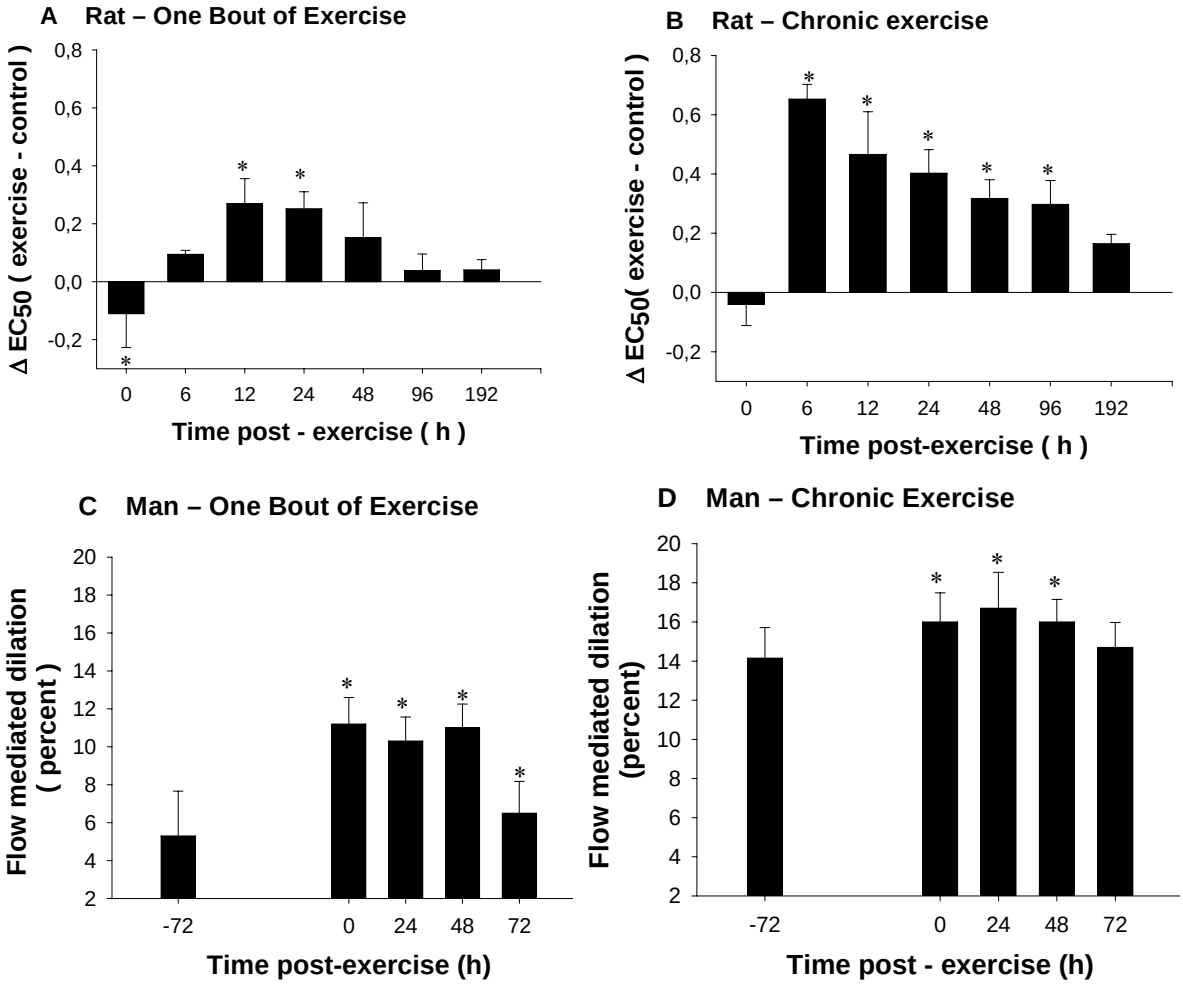

Figure 3. *Endothelial function determined by acetylcholine induced vasodilation in rat (panel A and B) and by flow mediated dilation in humans (panel C and D). After one bout of exercise in rat (panel A) a transient decrease was followed by a rapid increase in endothelial function also evident in humans (panel C). Chronic high intensity exercise in rat (panel B) and humans (panel D) also showed increased absolute relaxation.*

Regulation of eNOS-content and expression

In rats with low inherited VO_{2max} (LCR), arterial eNOS expression was markedly decreased, in line with the impaired NO-mediated vasorelaxation. Although exercise improved endothelial function in LCR up to the level of sedentary HCR, the eNOS expression was not up-regulated to the same extent. This strongly suggests that exercise promoted countermeasures (as discussed later) that restored endothelial function rather than reversing a

primary defect in eNOS of LCR. Failure to increase eNOS levels is consistent with the notion that exercise does not always increase eNOS levels[103], and that when it does, it exerts its effect in conjunction with restoration of lipid metabolism[209]. Endurance training did not fully restore triglyceride levels, and whether this underlies the failure of exercise to restore eNOS levels remains to be determined. One might also speculate that one or more shear stress responsive elements responsible for the transcription of the eNOS sequence are dysfunctional[44] and that the eNOS mRNA was unstable in the LCR. Additionally, as we measured the endothelial function in the distal part of the abdominal aorta, we measured the eNOS level in the proximal abdominal aorta, and we cannot exclude the possibility that this may be the cause of the mismatch between vasorelaxation and eNOS content since the level of eNOS is known to vary in the vascular system[110]. However, we do believe that this is unlikely since the distance between the segments of the abdominal aorta used for functional and molecular measurements were overlapping and the samples used for western blotting were pooled.

Hsp 90 was down-regulated in LCR as compared to HCR, but exercise restored it up to the levels of HCR, in line with improvements in endothelial function. Hsp 90 has a positive role in the regulation of signal tranduction with eNOS[13,62]. Hsp 90 binds directly to eNOS, augmenting NO production by inducing conformational adaptation of eNOS that renders it more readily phosphorylated by Akt[59]. Decreased Hsp 90 is on the other hand associated with a shift from NO to O_2^- and hydrogen peroxide production[146]. However, other possible molecular improvements that upregulate the bioavailability of NO after a single bout or long-term exercise are numerous. Long-term as well as one bout of exercise can upregulate the antioxidant defence, thus increasing the production and half life of NO[60]. Interestingly, the level of tetrahydrobiopterin, a protein that makes eNOS a NO producer instead of a O_2^- producer, was elevated after one bout of exhaustive exercise[121]. Also, augmentation of endothelial function following exercise training was associated with increased L-arginine transport in human heart failure[134].

Caveola density and caveolin-1

Regulation of NO production by shear stress is proposed to act through mechanotransducers such as integrins, ion channels, G proteins, and caveolae[176,177]. Activation of eNOS is a multifactorial process involving a balance of stimulatory and inhibitory protein/protein

interactions and phosphorylations in caveolae membrane compartments. eNOS is sequestered in these caveolae membrane compartments with agonist receptors such as acetylcholine-receptor, Ca^{2+} players, and signalling proteins[10,66,69,104,120,176]. Disruption of the caveola system profoundly impairs acetylcholine mediated relaxation[52,149]. Disturbances of the caveolae system have also been linked to the metabolic paradigm in vascular diseases such as atherosclerosis, leading to a failure of acetylcholine-mediated vasorelaxation similar to the one we reported in LCR[22,41,176,177]. Since decreased VO_{2max} is closely linked to decreased cardiac output and therefore also decreased shear stress[45,46,217], we hypothesized that decreased VO_{2max} would be associated with less shear stress responsive elements, such as caveolae. Consistently, we observed a decrease in the density of caveolae in LCR along with a 2-fold decrease in caveolin-1 (paper II). Exposing cultured endothelial cells to shear stress has been shown to increase the density of caveolae as well as the level of eNOS and NO[29,159]. Thus, we hypothesized that the arterial shear stress developed during exercise, would increase the caveolae density along with improvements in endothelial function. In accordance with this, exercise induced an increase in caveolae density along with an increase in caveolin-1, indicating that this pathway was a key element in the exercise-induced improvement of endothelial function in LCR.

A low level of caveolin-1 in aorta was associated with a relative endothelial dysfunction that was reversed by regular exercise training. Interestingly, caveolin-1 inhibits eNOS, which is clearly seen in the vigorous NO-mediated relaxation of acetylcholine stimulated arterial segments of Caveolin-1 (-/-) mice[52,149]. Previous work have shown no effect of exercise or chronic heart failure on the expression of caveolin-1 in aortic tissue[209], but an increase after administrating a high fat diet[191], in experiments involving aortacaval shunts[106] and exposing shear stress on endothelial cell cultures[29,159]. Since the augmentation of caveolin-1 was associated with an increased density of caveolae and improved endothelial function, one can speculate that the sequestration of eNOS in caveolae together with the muscarinic cholinergic receptor and the other members of its regulatory pathway may be the critical point necessary to restore the response to acetylcholine as the dissociation of caveolin-1 and eNOS is augmented during agonist mediated increase in Ca^{2+}- concentration[176,177].

Metabolic distortions and endothelial function

From earlier work we know that distortions of lipid metabolism, either primary[160,209] or secondary to diabetes[12], induce early perturbation of acetylcholine-dependant relaxation and

decrease eNOS expression in arteries. Although we did not measure cholesterol in our work, we know that oxidized low density lipoprotein (ox-LDL) decreases the level of eNOS[114] and it has been shown that LDL decreases the level of eNOS mRNA and protein in a concentration dependent manner[198]. In LCR, elevation in plasma triglyceride and free fatty acids (FFA) levels occurred in conjunction with endothelial dysfunction at an early age (paper I), supporting the hypothesis that this might cause decreased eNOS. Furthermore, the adipose derived cytokine TNF-α has detrimental actions upon the endothelium[158] and was up-regulated in adipose tissue of LCR. TNF-α has previously been shown to downregulate eNOS mRNA, protein, and activity in bovine and human arterial endothelial cells[4,107] along with destabilization of eNOS mRNA[212]. The failure to downregulate this adipokine by means of exercise, might cause the eNOS levels to remain at a low level (paper II). Interestingly, partial and full deletion of the eNOS gene increases susceptibility for high-fat diet-mediated arterial hypertension[37] and a phenotype of the metabolic syndrome[38,53], respectively. This suggests that a lower abundance of eNOS can be a molecular link between cardiovascular and metabolic disease, which is also consistent with the relative cardiovascular and metabolic distortions in untrained LCR as compared to HCR, where the eNOS level was 1/5 of HCR. However, whether the downregulation of eNOS is constitutive or due to transcriptional or posttranscriptional alterations, remains to be determined. Furthermore, early impairment of endothelial function is associated with only posttranslational changes of eNOS activity[91], and the eNOS level has been found to be downregulated in clinically relevant human atherosclerosis[95], indicating that the LCR may have progressed further into atherosclerosis, as compared to HCR. This is further supported by the fact that PPARγ, which is highly expressed in atherosclerotic lesions[157], was more evident in the endothelium of LCR. Exercise training did not change the immunoreactivity for PPARγ in the carotid artery of either strain of rats. This suggests that LCR are predisposed to atherosclerosis and this risk is not modifiable by 8 weeks of endurance exercise.

Hyperglycemia can induce superoxide anion generation in endothelial cells[185,211] that can lead to decreases in eNOS expression and activity, eventually reducing the production and half-life of NO. This might be the case for the male rats (paper I), but the lack of overt basal hyperglycemia in the female rats (paper II) suggests that the modest changes in blood glucose were unlikely to explain the alterations of endothelial function. However, hyperglycemia can be ameliorated by exercise[217], along with increased levels of SOD, a protein which will quench reactive oxygen species and increase the half life of NO[75].

Insulin's ability to vasodilate is well known, but whether this is initiated in the endothelium or smooth muscle, remains unclear[34]. Even though insulin levels were increased 2-fold (paper I) in LCR rats and insulin resistance was evident (paper II), they had hypertension and relative endothelial dysfunction compared to HCR rats. An imbalance between the pressor (sympathetic nerve stimulation, antinatriuresis, vascular hypertrophy) and vasodilator actions of insulin has been proposed as a link between insulin resistance and hypertension. According to this hypothesis there is a resistance to the actions of insulin on glucose uptake and vasodilation, but not to its pressor activities[7]. Although insulin-resistance was only marginally improved by exercise, the mean arterial blood pressure decreased 10 mmHg. However, this reduction in blood pressure can be explained by the improved endothelial function which results in decreased total peripheral resistance and afterload[72].

Cardiac function

Evidence of the Athlete's Heart

The athlete's heart is a hypertrophied heart with an increase in left ventricle volumes and enhanced pumping capacity. A high level of both intrinsic and acquired level of VO_{2max} was associated with the athlete's heart. Left ventricular weights scaled appropriately to body mass were 19 % higher in HCR vs. LCR (paper I). In both HCR and LCR, endurance exercise increased left ventricular weights and cardiomyocyte length significantly, but the increase is significantly more in HCR than in LCR. In normal Sprague Dawley rats, endurance training induced increases in left ventricular mass, cardiomyocyte length, and width (paper III). It is apparent from the present study and others[123] that longitudinal cardiomyocyte growth is sufficient to account for the effect of training on myocardial mass and provides a cellular mechanism to explain the eccentric ventricular hypertrophy that is often elicited by programs of aerobic exercise in humans and animal models of exercise. In figure 4, the relationship between VO_{2max} and left ventricular hypertrophy has an exponential form. This fits with Wagner's hypothesis that untrained subjects are demand-limited and that improvement in VO_{2max} early in a training period is due to peripheral factors, whereas fit subjects seems to be supply limited, i.e. most improvement in VO_{2max} is therefore due to increased maximal cardiac output. In healthy subjects[183], 8 weeks of endurance training improved VO_{2max} by 18 %, which was associated with increased stroke volume and enhanced contractility. Detraining athletes for 12 weeks however, led to a 20 % decrease in VO_{2max} along with decrease in stroke volume and left ventricular end diastolic dimensions[118]. We demonstrated a close correlation

between physiological hypertrophy and contractile function in isolated cardiomyocytes (paper I and III) (figure 5). Cessation of exercise led to a decrease in heart weights, reaching sedentary values after 2 to 4 weeks of inactivity. Despite this, cell-length remained significantly above that observed in controls after 4 week of inactivity, and this was the measure that was most closely correlated with the changes in VO_{2max} (paper III). Although no human data exist on exercised cardiomyocytes, significant reduction in cavity size and normalization of wall thickness has also been observed in detrained athletes[139], suggesting that the athlete's heart is also sensitive to training/detraining.

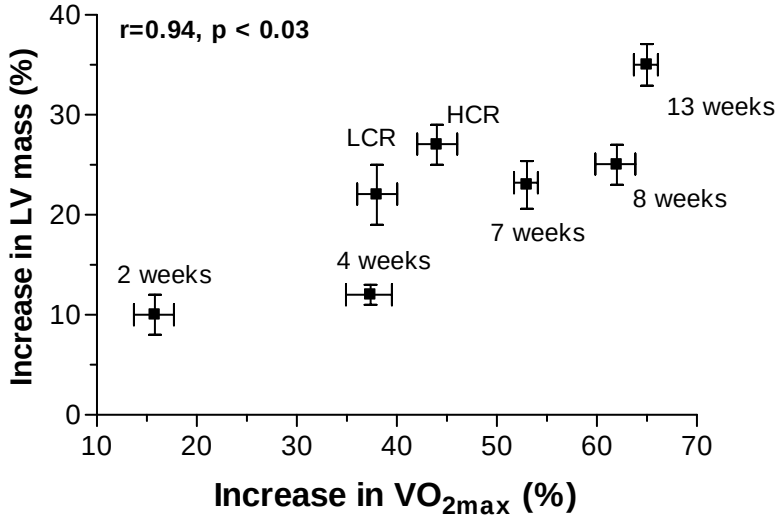

Figure 4*. Relationship between increases in VO2max (%) and left ventricular hypertrophy (%). Data are presented as mean ± SD from 100 rats. LCR; low capacity runners, HCR; high capacity runners. Data are from paper I and previous studies in our laboratory*[116,205]*.*

During the last 10-15 years, detailed studies of transcriptional, translational, and post-translational regulation have characterized a host of molecular mechanisms and signalling pathways associated with cardiomyocyte growth. A detailed description of these molecular mechanisms is beyond the scope of this work and is described thoroughly elsewhere[86].

Cardiomyocyte contractility

The level of VO_{2max} was closely related to cardiomyocyte contractile function. Cardiomyocytes from rats with a high VO_{2max}, both intrinsic and acquired, showed a greater degree of fractional shortening and had shorter relengthening time compared to those with a low VO_{2max}. These data are in line with Moore et al[123] and previous studies in our laboratory[204,205] showing an increased amplitude of shortening in cardiomyocytes from trained

animals, but differs with those of Laughlin[111] reporting no effect of training. Differences in training protocol, stimulation frequencies, and temperature used when stimulating the cardiomyocytes might explain these contrasting results.

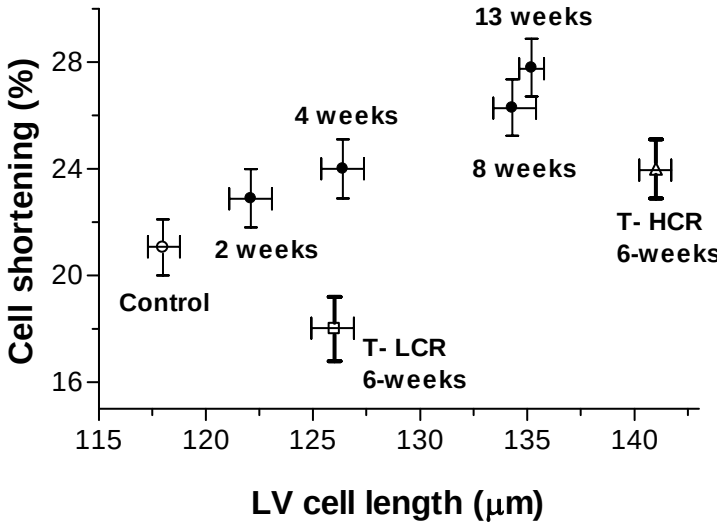

Figure 5*. Time dependent increase in cardiomyocyte length and maximal extent of shortening in cardiomyocytes isolated from endurance trained and sedentary rats. Each data point represents mean ± SD of 60 cells, 9 ±3 in each rat (n=6). In each cell data were calculated as the mean of 10 consecutive contractions after stabilization at 7 Hz. T; trained, HCR; high capacity runner, LCR; low capacity runner. Data are from paper I and previous studies in our laboratory[116,205].*

Previously it has been shown that training-induced elongation of left ventricular cardiomyocytes occurs in the absence of changes in sarcomere length[123]. Also, it appears that the changes in contractile function produced by endurance training are due in part to cardiomyocyte length-independent changes in contractile function. Several lines of evidence support this assumption. Schaible et al[171] demonstrated that endurance training elicited increases in end-diastolic volume, stroke work, ejection fraction, and mid-wall fractional shortening in the absence of changes in end-diastolic wall stress in perfused working rat hearts. Additionally, isometric force development by rat left ventricular papillary muscle is increased by endurance training[193]. Despite that, cell length remained significantly above what was observed in controls after 4 week of inactivity. Exercise-induced improvement in cardiomyocyte shortening regressed completely within 2 to 4 weeks of detraining (paper III).

Cardiomyocyte calcium handling

In line with increased rate of cardiomyocyte shortening and re-lengthening, we found an increased rate of Ca^{2+} release in systole and Ca^{2+} removal in diastole in trained normal Sprague Dawley rats (paper III). However, despite increased fractional shortening in normal healthy Sprague Dawley rats, the Ca^{2+} amplitude remained unchanged. These data suggest that training induced an increase in the Ca^{2+} sensitivity of the contractile element. Previously, it was shown that the increased Ca^{2+} sensitivity can be attributed to a higher intracellular pH observed at physiological stimulation frequencies[206,207]. Furthermore, permeabilized cells from trained rats shorten to a greater extent than sedentary cardiomyocytes in the presence of a constant buffered pH. These results indicate that the contractile proteins of the cardiomyocytes from trained rats have an increased intrinsic Ca^{2+} sensitivity compared to sedentary cardiomyocytes. The cellular basis for these changes is not known, but multiple biochemical alterations of the contractile proteins, including changes in the expression of troponin I and T isoforms, and increased alpha- myosin heavy chain expression [6,90,202]. Furthermore, Diffee et al[48,49] reports increased Ca^{2+} sensitivity in conjunction with increased expression of atrial myosin-light chain-1. Atrial myosin-light chain-1 has previously been shown to increase in human cardiac hypertrophy and has been associated with increased Ca^{2+} sensitivity to tension and increased power output.

A somewhat different pattern of Ca^{2+} handling was observed in isolated left ventricular cardiomyocytes from HCR and LCR rats. Intrinsically high VO_{2max} was associated with a similar adaptation in Ca^{2+} kinetics (time to peak and decay of Ca^{2+}) as in trained normal rats. HCR had a lowering of the diastolic and an increased systolic Ca^{2+}; thus HCR had an increased Ca^{2+} amplitude and more Ca^{2+} available for contractile work compared to that observed in cardiomyocytes from LCR rats. Furthermore, in this study (paper I), the increased cardiomyocyte shortening in both trained HCR and LCR was associated with a lowering of peak systolic and diastolic Ca^{2+}. Lower peak systolic Ca^{2+} transients have been reported earlier by Moore et al[123] and Wisløff et al[206,207], but not by others[111]. The reduction in peak systolic Ca^{2+} concentration in trained cardiomyocytes could be due to: (i) reduced Ca^{2+} released into cytosol via sarcolemma and SR; (ii) dilution of released Ca^{2+} into the sarcoplasm due to increased average cardiomyocyte volume; (iii) increased intracellular Ca^{2+} buffering capacity. The first two possibilities are unlikely since reduced Ca^{2+} influx or diluted cytosolic calcium concentration ($[Ca^{2+}]$) would reduce the Ca^{2+} binding to myofilaments and reduce contractility. The final possibility is feasible since only a small fraction of Ca^{2+} that is

released into and removed from the sarcoplasm during an excitation-contraction coupling cycle exists as free Ca^{2+} [181]. This adaptation to training is consistent with lower diastolic and systolic [Ca^{2+}] in trained cardiomyocytes. Tibbits et al[192,193] have demonstrated that Ca^{2+} binding sites increased by about 65 % in papillary muscle from trained rats. Penpargkul et al[140] reported enhanced Ca^{2+}-binding by cardiac SR from trained rats. Lower diastolic [Ca^{2+}] in trained cardiomyocytes could also result from enhanced sarcolemma ATP-dependent Ca^{2+} extrusion[142] and/or mitochondrial metabolism[20], thus effectively lowering the set point for Ca^{2+} regulation[31] in trained cardiomyocytes. Changes in myofilament Ca^{2+} affinity can dramatically affect amplitude and time course of the Ca^{2+} transient. The cardiotonic agent sulmazole increases myofilament Ca^{2+} binding affinity and peak myocardial force development, reduces peak systolic Ca^{2+} [23], and increases Ca^{2+} transient decay. Similarly, intracellular alkalosis increases cardiomyocyte shortening by increasing myofilament Ca^{2+} sensitivity. The accompanying Ca^{2+} transient is smaller in amplitude and shorter in duration[3]. We previously showed that trained cardiomyocytes have a significantly less acidic intracellular pH at high stimulus rates (>2 Hz)[207]. It is possible that the lower systolic [Ca^{2+}] after training is due to higher intracellular pH. However, this explanation is insufficient since intracellular pH is comparable below 2 Hz, yet trained cardiomyocytes shorten to a greater extent[207]. Without data on intracellular Ca^{2+} buffering capacities or Ca^{2+} flux, free [Ca^{2+}] cannot be directly related to the amount of Ca^{2+} released into the cytosol. Ca^{2+}-transient time courses were back to baseline values within 2 to 4 weeks of detraining and explain the regression of cardiomyocyte shortening in the same time period (paper III).

Although not measured in the present studies it is fair to speculate that the increased rate of Ca^{2+} removal can be explained by increased expression of SERCA2 as observed in previous studies in rats in our laboratory[206]. Thus, increased Ca^{2+} uptake capacity of the SR caused by increased SERCA2 expression could account for the increased rate of decay of the Ca^{2+} transient. In line with this, Ellingsen et al[54] demonstrated that differences in contractility and relaxation between exercise-trained and untrained hearts were significantly reduced by selective protein kinase inhibition. This is consistent with the notion that exercise-induced activation of Akt enhances cardiomyocyte contractility and relaxation by phosphorylation of proteins involved in Ca^{2+} handling, either directly or via interaction with other protein kinases. Exactly how this happens has not been fully investigated; compelling evidence includes increased channel function or phosphorylation in the presence of similar myocardial protein levels of L-type Ca^{2+} channels and ryanodine receptors, respectively[98,162]. Cardiac

specific over-expression of nuclear targeted Akt increased Ser-16 phosphorylation of phospholamban, corresponding to a larger phosphorylation of PKA, which also has Ser-16 as a target[162].

MAIN CONCLUSIONS

1. Diverging aerobic capacity, either inherited or acquired, correlates with improved metabolic risk profile and beneficial cardiovascular adaptations, both in the sedentary state and in response to exercise.

2. Selection for low versus high intrinsic aerobic capacity generated a different load of metabolic and cardiovascular risk factors constituting the metabolic syndrome. Our data clearly indicate that low aerobic capacity constitutes the physiological basis which predisposes the subject to clinical manifestations of disease such as the metabolic syndrome.

3. The current studies demonstrate that even in rats with inherited low aerobic capacity and metabolic syndrome, endurance training reduced cardiovascular risks insofar as it restored cardiomyocyte function, mitochondrial biogenesis in skeletal muscle, endothelial dysfunction, and visceral adiposity. However, it failed to reverse hypertriglyceridemia and lipogenesis in liver and fat tissue.

4. Cardiovascular adaptation to acute and regular exercise is highly dynamic. Upon detraining, most of the exercise-induced gain in VO_{2max} and cardiomyocyte function acquired over 8-12 weeks is lost within 4 weeks, whereas improvement in endothelial function was lost after a week of detraining. Furthermore, a single bout of exercise improved endothelial function for about 2 days with peak effect after 24 hours.

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