Ole Johan Kemi

On the Cellular Basis
of Aerobic Fitness

Intensity-Dependence and Time-
Course of Cardiomyocyte and
Endothelial Adaptations to
Exercise Training

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Innovation and Creativity

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ACKNOWLEDGEMENTS

Trondheim, August 2005

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Paper I

Ulrik Wisløff, Jan Helgerud, Ole Johan Kemi, Øyvind Ellingsen.

Intensity-controlled treadmill running in rats: VO_{2max} and cardiac hypertrophy. *American Journal of Physiology Heart and Circulatory Physiology* 2001;280:H1301-10.

Paper II

Ole Johan Kemi, Jan Pål Loennechen, Ulrik Wisløff, Øyvind Ellingsen.

Intensity-controlled treadmill running in mice: cardiac and skeletal muscle hypertrophy. *Journal of Applied Physiology* 2002;93:1301-9.

Paper III

Ole Johan Kemi, Per Magnus Haram, Jan Pål Loennechen, Jan-Bjørn Osnes, Tor Skomedal, Ulrik Wisløff, Øyvind Ellingsen.

Moderate vs. high intensity: differential effects on aerobic fitness, cardiomyocyte contractility, and endothelial function. *Cardiovascular Research* 2005;67:161-172.

Paper IV

Ole Johan Kemi, Per Magnus Haram, Ulrik Wisløff, Øyvind Ellingsen.

Aerobic fitness is associated with cardiomyocyte contractile capacity and endothelial function in exercise training and detraining. *Circulation* 2004;109:2897-904.

DEFINITIONS

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.

Ca^{2+}-induced Ca^{2+} release The subcellular events that initiate cardiomyocyte contraction. The inward L-type Ca^{2+} current stimulates the Ryanodine receptor to release Ca^{2+} from the sarcoplasmatic reticulum. The released Ca^{2+} induces myofilament contraction.

Detraining Complete withdrawal of exercise after a history of regular exercise training.

Endothelial function Includes several faculties of arterial endothelium; is in this thesis indicated by acetylcholine-induced nitric oxide generation in the endothelium that relaxes the vessel wall smooth muscle and induces vasodilation.

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

Maximal oxygen uptake ($V\text{O}_{2max}$) The highest oxygen uptake achievable during dynamic exercise with large muscle groups; the best single physiological measure of aerobic fitness.

Myofilament Ca^{2+} sensitivity The myofilament contractile response to any given $[Ca^{2+}]_i$ in the cardiomyocyte.

SUMMARY

Beneficial effects of exercise are closely associated with fitness and maximal oxygen uptake (VO_{2max}). Capacity for oxygen transport increases mainly by improved cardiac function, including larger chamber volumes, myocardial hypertrophy, and enhanced diastolic and systolic function. Higher arterial conductance, capillarity, and oxygen utilization in skeletal muscle also contribute. The present thesis investigates the cellular basis for cardiac and arterial effects; how they correlate with changes in VO_{2max} during exercise training at high or moderate intensity, and during detraining.

The studies show that:

1. Regular aerobic exercise training increases running performance and VO_{2max}, induces cardiac hypertrophy, and improves cardiomyocyte contractility and arterial endothelial function.
2. High intensity exercise is more effective than moderate intensity exercise to increase aerobic fitness.
3. VO_{2max} correlates closely with cardiomyocytes size, contractility, and calcium handling during adaptation in the first weeks of exercise, during detraining, and with different training intensities.

We conclude that:

1. Aerobic fitness is intimately related to cardiomyocyte size and function.

2. The magnitude of adaptation to training depends on exercise intensity.

3. Intensity emerges as an important determinant for beneficial effects of exercise training.

INTRODUCTION

Physical activity reduces, but does not currently prevent the Western Society epidemic of cardiovascular disease from either reaching global proportions or taxing public health and economy (6,30). Lack of physical activity and lowered level of fitness contributes to the world-wide rise of life-style related diseases as cardiovascular disease, metabolic syndrome, and diabetes (118). Better, affordable prevention and treatment strategies to improve wide-scale health are called upon. Regular physical exercise represents such a strategy (17,20). It improves cardiovascular function, health and quality of life, and mortality in patients and in those at risk of developing disease (11,18,62,65,140,143). A Cochrane meta-analysis found that exercise training reduces mortality by 27% in coronary artery disease patients (91). However, recommendations on physical activity in primary and secondary prevention of cardiovascular disease are diffuse (5,52) despite indisputable evidence of aerobic fitness as an important clinical reference point and target (93,136). In order to develop optimal exercise protocols to improve health, a sound understanding of the responsible cellular biology of improved cardiac and vascular function after exercise training is required. Thus, this thesis studies the relationship between aerobic fitness and cardiomyocyte and arterial function.

Aerobic Fitness is Closely Associated with Maximal Oxygen Uptake

The single most important physiological measure of aerobic fitness in both health and disease is maximal oxygen uptake (VO_{2max}), which refers to the maximal rate at which oxygen can be transported from ambient air to peripheral working skeletal muscles, where it is utilized by intracellular metabolic processes (160,176,177). Work economy, i.e. oxygen cost of given work or ratio between power output and oxygen uptake (VO_2), and lactate threshold, i.e. highest VO_2 or power output without excess congestion of lactate, also contribute to aerobic fitness, but considerably less (77,126,145).

Determinants of Maximal Oxygen Uptake

Analytical modeling (26,47,176,177) and laboratory approaches (154,156) assert that cardiac output and arterial conductive and redistributive capacity are rate-limiting to aerobic exercise in every individual. A majority of studies indicate that VO_{2max} is by large determined by the

cardiac pumping capacity, as the main drop in oxygen partial pressure occurs between the pulmonary and skeletal muscle capillaries (160,176,177). This implies a regulatory role for arterial conductance. In contrast, pulmonary work and the problem of lung perfusion/diffusion mismatch is only rate-limiting to well-trained individuals, whereas skeletal muscle diffusion and metabolic flux appears rate-limiting only when convective supply surpasses peripheral demand, such as in unfit subjects or during work with only a small muscle mass (178). All things considered, the likelihood is that there is no sole determinant of $V\text{O}_{2\text{max}}$, but rather a complex chain of intertwined elements working in concert (154,176,177).

Intensity of Exercise Determines Outcome

Although defining studies of healthy individuals still are lacking, clinical trials point to the superiority of high aerobic intensity over low-to-moderate intensity to gain full effect of a training program (2,89,101,157,170). The emergence of $V\text{O}_{2\text{max}}$ as a continuum from health to disease supports this notion (93,136). Cardiovascular adaptations may therefore rely on the exercise intensity during long-term regular training programs also in healthy individuals, especially since it was demonstrated that stroke volume in well-trained athletes increases continuously with increasing intensity up to maximal levels at or around peak aerobic exercise intensity (59,194).

Exercise Training Induces Cardiomyocyte Hypertrophy

Adaptive growth of the cell in response to regular exercise (physiological hypertrophy) usually involves proportional growth in length and width (83,128,129). This corresponds with increased ventricular weights (8) and the phenomenon of the athletes' heart, manifested as increased chamber volumes and mass, and increased thickness of the left ventricle wall (146,147,150). Both increased gene transcription and translation seem to underlie physiological hypertrophy (25,102). In contrast, abnormal growth in response to pressure and/or volume overload characterizes pathologic hypertrophy, and involves early longitudinal cell growth followed by a later stage of proportional length and width growth (38,83). Hypertrophy in heart failure is normally linked to reprogramming into a fetal gene program expressing embryonic growth cascades (28).

Cardiomyocyte Contractile Function

A central feature of regular exercise training is improved systolic and diastolic function and larger cardiac output (50,59,162,186). Exercise training over months usually improves cardiomyocyte shortening and rates of contraction-relaxation (129,187,188), providing a cellular basis for global function. In contrast, heart failure induces cardiac dysfunction with reduced ejection fraction and lower cardiac output, which also is associated with impaired cardiomyocyte contractile function in both man and rodent (22,38,41,66,109,110,148,165,174, 182,187). Overall, the differences that occur between species seem to be quantitative, rather than qualitative (14,15,167). Experimental studies of cellular effects of regular exercise training appear therefore translatable to humans, also when exercise training is applied therapeutically in heart failure and cardiovascular disease. Such studies suggest restored contractility and attenuated pathological growth as important cellular mechanisms for the beneficial effects of physical activity in heart failure (187,190,192).

Cardiomyocyte Contractile Machinery

Cardiomyocyte sarcomere units composed of mainly actin and myosin filaments constitute the contractile protein machinery, together with tropomyosin and the troponin complex. When Ca^{2+} binds to troponin C, the complex changes conformation and troponin I moves to allow actin-myosin coupling, while troponin T attaches the complex to tropomyosin. Next, myosin heads bind to actin and flex to slide the filaments in opposite directions and cause shortening; hence, the process is termed the sliding filament model. Upon release of Ca^{2+} from troponin C, the machinery returns to its innate position and awaits the next contractile cycle. Titin, myosin binding protein C, M-protein, myomesin, nebulin and nebulette, and α- and β-actinin serve supporting and regulatory functions (14,15).

Cardiomyocyte Excitation-Contraction Coupling and Calcium Handling

The rapid transient increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) evoke global cell shortening as described above. Conversely, the Ca^{2+} decay causes relaxation. Details of the subcellular events regulating Ca^{2+} induced Ca^{2+} release are illustrated in figure 1.

Sarcolemma

ATP

Ca^{2+}

DHPR

RyR2

Ca^2

3 Na$^+$

NCX

Ca^{2+}

SR

PLN

SERCA2a

Ca^{2+}

Ca^2

NCX

Ca^{2+}

3 Na$^+$

Ca^2

Na$^+$

Ca^{2+}

Ca^2

Mitochondrion
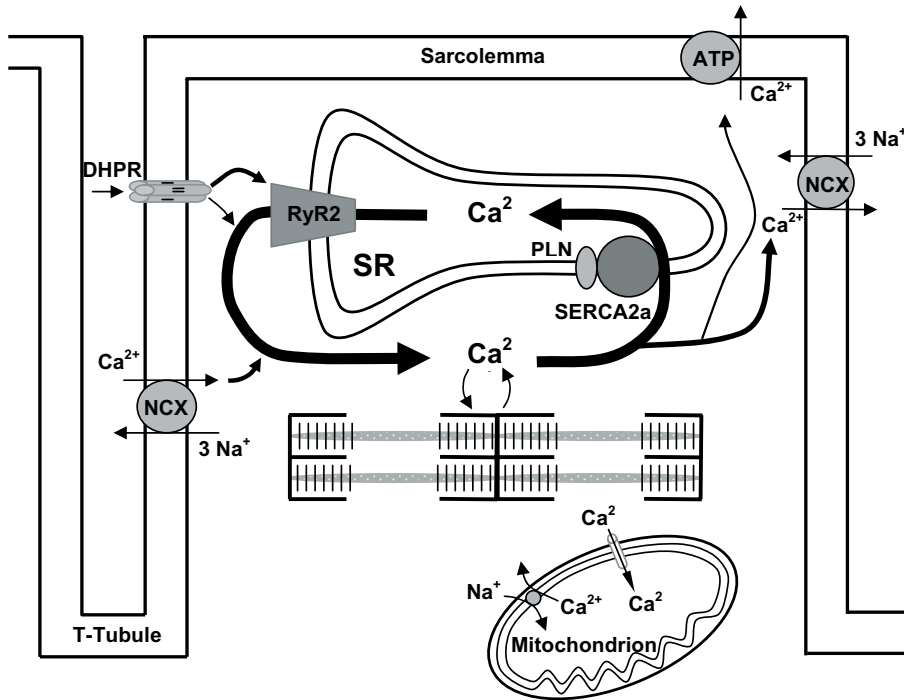
T-Tubule

Figure 1. Excitation-contraction coupling in ventricular myocytes. DHPR: L-type Ca^{2+} channel; NCX; Na$^+$/Ca^{2+} exchanger; T-Tubule: Transverse tubule; RyR2: Ryanodine receptor-2; SR: Sarcoplasmatic reticulum; PLN: Phospholamban; SERCA2a: SR Ca^{2+} ATPase-2a. Modified from Bers (14).

Depolarization allows a small entry of Ca^{2+} through the dihydropyridine receptor (DHPR; L-type Ca^{2+} channel) and through reverse mode Na$^+$/Ca^{2+} exchanger (NCX). The resulting [Ca^{2+}]$_i$ increase stimulates the ryanodine receptor-2 (RyR2) to release mM-order of Ca^{2+} from the Sarcoplasmatic Reticulum (SR). The co-location of the inward Ca^{2+} channels in transverse tubules and the RyR2 complex in SR secures a fast and efficient coupling, whereas RyR2-embedded proteins as FKBP12.6 regulate the open probability of the RyR2 to prevent diastolic leakage. Localized Ca^{2+} release (Ca^{2+} sparks) coordinate and generate the [Ca^{2+}]$_i$ transient that induces contraction (60,104,112). Post-contraction, the main bulk of Ca^{2+} is re-sequestered (Ca^{2+} decay) by the SR Ca^{2+} ATPase-2a (SERCA2a), which activity is closely regulated by phospholamban (PLN). Thus, SERCA2a recharges the SR Ca^{2+} load, whereas normal mode NCX and plasma membrane Ca^{2+} ATPases translocate Ca^{2+} out of the cell to the extracellular space. This is the prevailing mechanism of cell contraction-relaxation

(14,15,153), although alternatives relying on other stimuli have been suggested (48,51,80), and rebutted (15,94,173).

Intracellular Calcium Controls Myocyte Contractility

Studies of excitation-contraction coupling and Ca^{2+} cycling after exercise training have demonstrated that increased levels of SERCA2a and PLN at least partly explain why Ca^{2+} handling improves and $[Ca^{2+}]_i$ transport rates increase with regular exercise (188). Studies of other subcellular factors that may improve cell contractility are sparse, but clues are provided from manipulative and mechanistic studies (27,35,76,111,119,121,151). Thus, exercise training improve subcellular Ca^{2+} events that account for larger cardiomyocyte shortening and higher contraction-relaxation rates, although a complete cellular perspective of the underlying biology has not yet been provided.

In contrast, abnormal excitation-contraction coupling and depressed Ca^{2+} handling provide mechanisms for cardiomyocyte contractile dysfunction (60,66,68,104,109,133,148,163,182). Reduced SERCA2a levels (54,76,84,149) and inhibited activity due to decreased phosphorylation of PLN (16,105) reduces the SR Ca^{2+} cycling ability to cope with higher heart rates, and may distort normal force-frequency relationships (38,68) and thus Frank-Starling mechanics. The importance of SERCA2a was further documented by adenoviral gene therapy; restoring SERCA2a in cardiomyocytes corrected abnormal Ca^{2+} handling and contractile dysfunction (41,42), whereas targeted overexpression enhanced contractility in transgenics (9). Reduced SERCA2a levels may be compensated by enhanced phosphorylation of PLN (36). However, close intrinsic control is needed to maintain balanced Ca^{2+} cycling (172).

Myofilament Calcium Sensitivity

Regular exercise training increases myofilament Ca^{2+} responsiveness (46,188). Thus, increased cell shortening may not always correspond to altered bio-availability of Ca^{2+}. The biological basis for altered Ca^{2+} sensitivity is not well defined yet, but modification in the contractile machinery, e.g. troponin subunits and myosin light chains have been proposed (7,

45,100,137). However, another plausible explanation to increased cell contractility may be changed cooperation of actin and myosin or other contractile proteins, independent of Ca^{2+}. After myocardial infarction and in heart failure, the role of myofilament Ca^{2+} sensitivity response is unsettled, as results range from decrease via no change to increase (67,69,70, 109,148,164), despite improved clinical outcome of Ca^{2+} sensitizers (75). It may be that cellular changes affecting Ca^{2+} sensitivity are adaptive in exercise training, and compensatory rather than causative in heart failure, in order to increase or preserve contractile capacity.

Arterial Endothelial Function

Improved arterial endothelial function with exercise training improves conductance of blood flow, and is associated with depressed atherosclerosis due to inhibition of leukocyte adhesion and invasion, less platelet aggregation and adhesion, and suppressed smooth muscle proliferation (3,23,73,97,179). Since these effects depend upon endothelial release of the vasoactive and anti-atherogenic agent nitric oxide (NO), it appears that measuring NO-mediated vasorelaxation is indicative of endothelial function (99,124,138,189). In this thesis, endothelial function is therefore indicated by acetylcholine-induced vasorelaxation (see below); higher artery relaxation is interpreted as improved endothelial function. Endothelial dysfunction represents abnormalities in the pathways that regulate the level of NO. Other vasoactive agents released from the endothelium are endothelium-derived hyperpolarizing factor, prostacyclin, angiotensin II, thromboxane, prostaglandin, and endothelin (57,87,139).

Acetylcholine Stimulates Nitric Oxide-Mediated Vasorelaxation

The bio-availability of NO is mechanistically linked to artery relaxation and endothelial function, as illustrated in figure 2. NO is a small uncharged radical compound produced by oxidation of the terminal guanidino nitrogen of the amino acid L-arginine. The process is catalyzed by the constitutive endothelial isoform of NO synthase (eNOS), after stimulation by acetylcholine-binding to muscarinic receptors. Next, NO enters smooth muscle cells and initiates the signal cascade that ultimately decreases $[Ca^{2+}]_i$ and induces vasorelaxation (23,138,139). Besides acetylcholine, shear stress, bradykinin, ATP, ischemia, and a large number of extra- and intracellular factors may also mediate NO production (23).
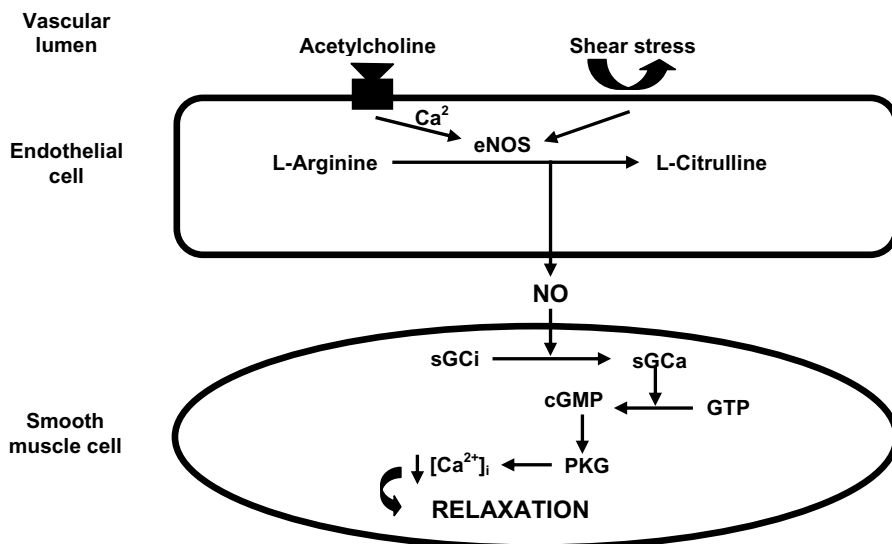
Figure 2. Acetylcholine-induced nitric oxide (NO)-dependent arterial relaxation pathway. eNOS: endothelial NO synthase; NO: nitric oxide; sGCi: inactivated soluble guanylate cyclase; sGCa: activated soluble guanylate cyclase; GTP: Guanosine triphosphate; cGMP: cyclic 3`,5`-guanosine monophosphate; PKG: cGMP-dependent protein kinase. Modified from Walther et al (179).

Higher level of NO in the smooth muscle cell induces relaxation and is an important cause of improved endothelial function after exercise training (71,179). Although endothelial function is associated with aerobic fitness (97,189), conflicting results appear during exercise training in normal individuals, from increased to unchanged and decreased endothelial function (13,29, 95,99,117,123,124). In endothelial dysfunction, acetylcholine fails to induce normal relaxation, and may in fact induce a paradoxical vasoconstriction by binding to cholinergic receptors on the smooth muscle cell surface (115). Thus, endothelial (dys)function is linked to cardiovascular health. Recent studies link improved cardiovascular health in cardiovascular patients to corrected endothelial function due to regular exercise training (3,72,73,97,106), partly due to enhanced phosphorylation of eNOS to restore NO-levels (71). Hence, endothelial dysfunction reduces the levels of NO and has been identified as an independent risk factor and marker of morbidity and mortality (74,161).

OBJECTIVE

The main focus of the present study was to investigate the cellular basis for cardiac and vascular contributions to aerobic fitness (VO_{2max}).

The specific aims of the studies were to:

1.   Develop valid and reproducible procedures to determine VO_{2max} during treadmill running in rats and mice.

2.   Establish long-term intensity-controlled exercise training in rats and mice that yield robust cardiovascular effects mimicking adaptation in humans.

3.   Determine how intensity of exercise influences VO_{2max}, cardiomyocyte hypertrophy, contractility and Ca^{2+} handling, and arterial endothelium-dependent relaxation.

4.   Determine the time-course of VO_{2max}, cardiomyocyte hypertrophy, contractility and Ca^{2+} handling, and endothelial function when high-intensity exercise training is implemented and withdrawn.

5.   Determine the relationship between cardiomyocyte contractile capacity and arterial endothelium-dependent relaxation on one side and VO_{2max} on the other side.

METHODOLOGICAL CONSIDERATIONS

Animal Models

The objective was to correlate VO_{2max} with cardiomyocyte and endothelial function. This requires access to viable cells and tissues and a close control of the intensity and time course of exercise training. Hence, procedures for exercise training and testing of VO_{2max} were developed and evaluated in animal models as detailed in papers I and II, since similar studies in humans are close to impossible. For both rodent models, availability and costs were favorable.

Rat

Adult female Sprague-Dawley rats were used in studies I, III, and IV, as they are previously well characterized in our lab (109,110,187,188) and elsewhere (43-46,190-193). Physiological body growth is less pronounced in females; thus, data related to body mass are less confounded than in males. As discussed below, this does not preclude gender-specificity on cardiac or vascular adaptations (63,142), which may affect cellular dynamics. As Sprague-Dawley rats come from an outbred strain, the genetic variability is preferably larger than in inbred animals and validates the results to a wider biological background. Moreover, the effects of anesthesia in this strain are well-known from previous experience in our laboratory (108-110).

Mouse

To expand future investigations to specific molecular and genetic targets via transgenic and gene-modified models, we extended our exercise model to adult mice. Thus, the first mouse training study from our laboratory is reported here (paper II). The C57BL/6J strain is inbred and more genetically homogenous than outbred animals. Hence, it is extensively used for pharmacological and physiological studies. Even though a recent report described it as inferior to other mouse strains with regard to running performance and exercise adaptability (122), responses in study II were fairly robust.

Exercise Training Program

To maximize cardiovascular adaptations, we chose the custom-made inclined treadmill running model that allows close control of exercise intensity, as our preferred model of exercise. The exercise sessions throughout the investigations were carried out at 85-90% of VO_{2max}, interspersed by short periods of milder intensity, except in paper III, in which moderate intensity at 65-70% of VO_{2max} also was employed to study cellular adaptations to different intensities. Uphill treadmill running is a full-body exercise that taxes the cardiovascular system and VO_2 maximally, and reduces running speed, which otherwise would have the potential to intrude reaching maximal work intensity (see data in papers I and II). Although this model may also cause stress reactions given the electrical grid (130), the physiological adaptations by large mimic those in humans (129,188). In comparison, other available exercise models as voluntary wheel running, fixed speed treadmill running or swim-training in a water tank (8,33,79,129), give little or no control of the amount or intensity of the work performed. In particular, forced swimming probably involves more stress.

The light-dark cycle of the animals was adjusted to accommodate exercise sessions during the animals' dark cycle, as normal behavior pattern summons activity at night. Finally, animals were rewarded chocolate after each exercise session as reinforcement to encourage treadmill running (158). To counteract bias, sedentary animals were also given the same amounts of chocolate.

Testing of Oxygen Uptake

To monitor training-induced adaptations and adjust treadmill running speed to maintain the desired relative exercise intensity, VO_{2max} was measured during treadmill running to exhaustion at the start of every training week. Running economy, i.e. VO_2 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 protocols for measuring VO_2 in rats and mice are detailed in paper I and II, respectively. The methods have been found reproducible through several studies in our laboratory in both normal healthy and heart failure rats, and in rats genetically selected and bred for either high or low aerobic capacity (papers III and IV;

187-189). Since paper II, we have reproduced the test and training protocols for mice in later unpublished studies. The principles behind the VO_{2max} test resemble those from human subjects (181,195) and confirm that VO_{2max} is integral for evaluating physical capacity and integrative function.

Anesthesia

The normal method for sacrificing animals was with diethyl ether anesthesia, which provides a quick comatose, but maintains a stable cardiac function that is vital for harvesting cardiomyocytes. Intraperitoneal injections with ketamine and xylazine provided anesthesia for echocardiography; a mixture widely used for echocardiography recordings of both rats and mice because of the well-defined cardiovascular effects (88,107,169). Ketamine provides analgesia, sedation, and amnesia. It may affect hemodynamics by increasing blood pressure and cardiac output. Hence, the lowest possible dose was used to balance depressive and stimulatory effects to secure adequate sedation. Following echocardiography, the animals were observed until awakening.

Echocardiography in Rats and Mice

Global adaptations on cardiac morphology and work performance were evaluated by echocardiography, as described in papers II and III. 2-dimensional M-mode long-axis recordings providing 250 frames per second were used to evaluate left ventricular dimensions, whereas pulsed-wave Doppler recordings at 500 Hz were used to assess flow characteristics, according to recommendations from the American Society of Echocardiography (159). From each recording, 5-10 consecutive cardiac cycles were averaged. Although echocardiography is fairly well established as a valid tool to assess left ventricular dimensions and function in the rat myocardial infarction model (88,107,108,166), its feasibility to evaluate training-induced cardiac adaptations is less certain, probably due to a smaller window of changes. In our hands, echocardiography was less sensitive than single cell measurements to detect hypertrophy and different contractile function between sedentary and trained (papers II and III; 188). This is consistent with the large number of animals needed to detect biologically important changes by standard equipment; ~50-60 individuals

would be needed to detect significant differences of 15% (α 0.05, β 0.8) of left ventricular size between different mouse groups in standard M-mode (31).

Cardiomyocyte Isolation

Cardiomyocytes were isolated to study cell size and function, as detailed in papers I and II. The procedures are modified from previous protocols for rats (82) and mice (103). Due to discontinued delivery of Joklik's minimum essential medium used in I and II to isolate left ventricular myocytes, a slightly different solution replaced the isolation medium. Thus, studies III and IV proceeded with Krebs Henseleit Ca^{2+} free solution, but with similar concentrations of the proteolytic collagenase type II and similar introduction to Ca^{2+} to ensure Ca^{2+} tolerance after isolation, which was performed in absence of Ca^{2+} to separate cells from each other. Our laboratory has extensive experience with both Krebs Henseleit and Joklik's solutions, and no differences in cell yield and intrinsic properties were noted throughout the studies. However, the batch of collagenase was an important source of variation in cell yield. We also noted that different batches of laminin caused some variation in myocyte attachment to the cover-slips. The number of left ventricular myocyte nuclei of the rat is estimated to ~33 · 10^6, whereas our average cell yield was ~3-4 · 10^6 from healthy left ventricles; thus, a recovery rate of ~10%, given that most cardiomyocytes are mononucleate; some may be binucleate (15). The percentage of rod-shaped cells was consistently ~70-75%. Only viable rod-shaped cells without obvious cellular damage were selected for measurements of dimensions, and only undamaged cells that also responded to electrical stimulation were chosen for measurements of contractility and $[Ca^{2+}]_i$ transients. Cells that failed to complete the stimulation protocol during the experiment were excluded from analysis. Exclusion rates were similar in all groups. In every experiment, cells from two different groups were isolated simultaneously, to maintain a balanced design and avoid systematic variation.

Electrical Stimulation of Cardiomyocytes

Electrical field stimulation is a standard and widely used means to study contractile function and Ca^{2+} dynamics in isolated cardiomyocytes, as it effectively characterizes cellular adaptations of the heart to exercise training, heart failure, and other diseases (68,109,110,

129,148,151,172,187-189). As a measure of contractile function, fractional shortening was measured during continuous perfusion with HEPES solution at physiological [Ca^{2+}] and 37˚ C. The magnitude of shortening was normalized to diastolic cell length, whereas changes in velocities of contraction and relaxation were indicated by time to half and peak shortening, or time from peak shortening to half-relaxation. [Ca^{2+}]$_i$ ratio was measured simultaneously.

Fluorescence and Edge-Detection Microscopy

In order to study contractility and intracellular Ca^{2+} handling and homeostasis (III and IV), cardiomyocytes were mounted on an inverted microscope with the optical train converted to epifluorescence, including a xenon arc 75 W excitation source, and fluorescence emission detection with a photometer and photomultiplier tube. Capture-and-analysis software handled the generated data. The extent of cell shortening and the rates of cell shortening and relaxation were measured by a sideport camera and video edge-detection from the light/dark contrast of the cell edge against background. Contractility and [Ca^{2+}]$_i$ transients were sampled simultaneously through an Fl 40X/ 1.3 NA oil immersion objective, while cells were stimulated by 5 ms and 1-10 Hz electrical field bipolar pulses; bipolarity to avoid electrolysis. A 500 Hz rotating optical chopper and bandpass filters excited the Fura-2/AM loaded cells by alternatively near-ultraviolet 340 nm and 380 nm wavelengths, whereas 510 nm emission was counted to allow ratiometric evaluation of [Ca^{2+}]$_i$ transients. When excited at 340 nm, fluorescence emission increases with increasing [Ca^{2+}]$_i$, whereas at 380 nm excitation, emission decreases with increasing [Ca^{2+}]$_i$. Thus, ratioing minimizes possible aberrations of uneven dye loading, leakage, photobleaching and unwanted compartmentalization of Fura-2 in the cell (Grynkiewicz et al 1985). Because of uncertainty of calibration procedures and a non-linear relationship between the ratio and [Ca^{2+}] (53), we used ratios without converting to [Ca^{2+}]$_i$ for further analyses.

For detection of cell size, a large number of cells were video taped through either a Fl 20X/0.4 NA or Fl 40X/0.7 NA objective, whereupon 100-150 random cells per rat and 170-250 random cells per mouse were measured for length and midpoint width. Cells without visible damage were selected and calibrated by a stage micrometer. The standard deviations for cell length

and width from a pool of sedentary rat cardiomyocytes were found to be ±13 µm and ±3 µm, respectively, and ±11 µm and ±4 µm in mice.

Cardiomyocyte Calcium Sensitivity Index

To study how the contractile properties of the cell react to a given amount of available Ca^{2+} (67,70,148), a Ca^{2+} sensitivity index was calculated as the ratio between fractional shortening and the amplitude of the $[Ca^{2+}]_i$ ratio from diastole to peak systole. This is not a direct measure of the myofilament sensitivity to Ca^{2+}; however, previous studies have established this as an adequate index (109,188). Direct evaluation would require permeabilization of the plasma membrane, which would compromise contractile function, or mounting the cell to force transducers to trace loaded shortening in order to determine the power production of the given Ca^{2+} (46). Moreover, indirect measurements of Ca^{2+} sensitivity imply that the intracellular environment in terms of e.g. pH is not controlled, which may affect myofilament Ca^{2+} sensitivity, whereas the time lag between peak $[Ca^{2+}]_i$ transient and peak shortening of the cell may to some degree distort the evaluation. Nonetheless, the Ca^{2+} sensitivity index results indicate that myofilament contractile dependence of Ca^{2+} in the isolated cardiomyocytes adapts to chronic exercise.

Arterial Endothelial Function

To assess endothelial function, acetylcholine-mediated endothelium-dependent vasorelaxation was measured by mounting the right common carotid artery onto a force transducer and a micrometer in a continuously equilibrated Krebs solution at 37° C, as detailed in papers III and IV. The protocol was adapted and modified from studies of piglet femoral arteries (168), and represented the first vascular assessments our laboratory performed in rat (paper III). After the first study, it became evident that, after pre-contractions with the α-agonist phenylephrine, higher concentrations of acetylcholine were required to ensure maximal relaxation in all artery segments (see paper III, figure 5). This was effected in the succeeding study (IV), whereas conventional curve-fitting methods were used to calculate maximal relaxation levels in the artery segments that did not level off in study III.

Acetylcholine induces endothelium-dependent vasodilatation via muscarin receptors. To ascertain endothelium-dependence in the tension measurements, arterial segments from each animal were also exposed to L-NAME, an L-Arginine competitor that inhibits the L-Arginine-NO pathway and thus blocks NO-production. Arterial segments were also exposed to Na^+ nitroprusside; an endothelium-independent NO-donor. The prevailing NO synthase in the arterial wall is eNOS (71,74), which L-NAME inhibited effectively and Na^+ nitroprusside bypassed by evoking maximal relaxation independent of endothelial NO. The interventions confirmed that arterial vasodilatation induced by acetylcholine were endothelium-dependent. Indomethacin was added to all experiments to exclude confounding by cyclooxygenase-mediated prostaglandin and thromboxane-conversion from arachidonic acid.

Allometric Scaling

Since studies I and II involved both sexes and every study involved long-term follow-up, body mass might influence the results. Thus, allometric dimensional scaling was applied to appropriately normalize VO_2 and cardiac and skeletal muscle weights to body size. Traditionally, data were usually divided by blank body mass. However, this may lead to underestimation in heavier subjects (10,37). Measuring lean body mass is difficult and would require body scanning to distinguish different tissues. At the time of our studies, scaling VO_2 to body mass raised to the power of 0.75 (exponent b) occurred as the best available method, in line with theoretical modeling and empirical studies over a wide range of body masses and species (171). Recent investigations published after the present studies, have estimated different mass exponential values, especially at maximal metabolic rate as indicated by VO_{2max}. By re-examining 34 eutherian mammalian species ranging 7 g to 500 kg from a large body of published data (31 original reports, including paper II), it was found that VO_{2max} should be reported in relation to a body mass with the exponent b 0.872 (± 0.029, 95% confidence limits 0.813-0.932; 184,185). However, the exponent b may differ between athletic and non-athletic phenotypes, as a tight connection was found between how VO_{2max} relates to body mass and aerobic capacity in the skeletal locomotor muscles. The allometric cascade was also examined with a different multiple-cause model, in which the relative contributions along a detailed cascade were assessed, from pulmonary ventilation and alveolar-to-arteriolar

oxygen diffusion, to skeletal muscle fiber, Ca^{2+} pump and myosin ATPase activities at the receiving end (81). With this approach, the exponent *b* was found to be 0.84-0.92 during maximal metabolic rate, and 0.74-0.77 during basal metabolic rate. However, when re-analyzing our VO_{2max} data with the mass exponent 0.872, the conclusions remained the same, since body masses remained similar between trained and sedentary animals.

Following the best current approximation, cardiac weights were related to body mass with the exponent 0.78 (10). In paper II, skeletal muscle weights were also measured. To the best of our knowledge, no previous studies could advice on how to scale appropriately. Therefore, we calculated the exponent *b* from the present population by allometric equations. This approach yielded the exponent *b* 0.75, almost similar to the cardiac exponent. However, care should be granted, as defining a valid exponential power usually requires a much larger pool of data (90).

Statistical Analysis

As each study operates with a limited number of animals per group, data were analyzed by non-parametric procedures and complemented by one-way and repeated measures ANOVA, where appropriate. In papers III and IV, we investigated which cellular factors best corresponded to VO_{2max}, to identify the cellular determinants of aerobic fitness. Such attempts were performed by simple univariate and multivariate linear regression analyses. Although this approach identified several cellular features, variables that did not reach statistical significance may still be biologically important. The backward stepwise model was chosen to include all independent variables and then remove insignificant ones one at the time until a final model with significant cellular contributors was achieved. However, the same variables were identified using the forward stepwise model.

SUMMARY OF RESULTS

PAPER I   **Intensity-controlled treadmill running in rats: VO_{2max} and cardiac**

                hypertrophy

The objectives of this study were to develop and evaluate experimental procedures for

determining VO_{2max} during treadmill running in rats of both sexes, and to determine the effects

of long-term high-intensity treadmill exercise training at 85-90% of VO_{2max}.

Evaluation of Test Procedures

1.   Repeated measurements of submaximal running yielded a high reproducibility:

        VO_2: r = 0.96, coefficient of variation = 4%.

        Heart rate: r = 0.98, coefficient of variation = 1.5%.

2.   Optimal treadmill inclination for measuring VO_{2max}: 25° (47%).

3.   Respiratory exchange ratio > 1.05 and blood lactate > 6.0 mM confirmed exercise

        level at or close to exhaustion.

4.   Linear relationship between VO_2 and heart rate was found during increasing treadmill

        running velocities; maximal heart rate required higher velocities than VO_{2max}.

5. VO_2 leveled off (VO_{2max}) during treadmill running, despite increased running velocity.

Effects of Regular Exercise Training

1.   Exercise training induced an athletic phenotype resembling human cardiopulmonary

        adaptations to exercise.

2. VO_{2max} and maximal aerobic running velocity increased 60-70%.

3.   Resting and maximal oxygen pulse increased 25-30%, indicating increased stroke

        volume.

4.   Exercise training improved running economy during submaximal treadmill velocities:

        Oxygen cost decreased ~16%.

        Heart rate decreased 11-13%.

        Respiratory exchange ratio decreased ~5%

5.   Exercise training induced myocardial hypertrophy; right and left ventricular weights

        increased 23-34%, while left ventricular myocyte length increased 6-12%.

PAPER II **Intensity-controlled treadmill running in mice: cardiac and skeletal muscle hypertrophy**

The objective was to extend the experimental treadmill running protocols into a mouse model. Thus, the study established valid and reproducible protocols for determining aerobic capacity, and protocols for exercise-induced cardiopulmonary adaptations in mice of both sexes similar to those reported in rats and humans.

Evaluation of Test Procedures

1. Repeated measurements of submaximal VO_2 yielded a high reproducibility: r = 0.98, coefficient of variation = 9.1%.

2. Optimal treadmill inclination for measuring VO_{2max} was 15-35°; highest respiratory exchange ratios were obtained at 25°.

3. VO_2 increased linearly with increasing treadmill velocity, until leveling off at VO_{2max}, despite increasing running velocity. Respiratory exchange ratio > 1.0 confirmed exercise level at or close to exhaustion.

Regular Exercise Training

1. Regular exercise training in mice of both sexes induced a similar athletic phenotype as in paper I, and resembled human cardiopulmonary adaptations to exercise.

2. VO_{2max} increased 30-50%; maximal aerobic running velocity increased ~70%.

3. Exercise training improved running economy during submaximal treadmill velocities:
    Oxygen cost decreased 19-25%.
    Respiratory exchange ratio decreased ~8%

4. Exercise training induced myocardial hypertrophy; left and right ventricular weights increased 19-29% and 12-17% in females and males, respectively. Left ventricular myocyte length and width increased ~20-30% in both sexes.

5. Echocardiography indicated cardiac hypertrophy.

6. Extensor digitorum longus and soleus skeletal muscle weights increased 12-18%

PAPER III   **Moderate vs. high exercise intensity: differential effects on aerobic fitness, cardiomyocyte contractility, and endothelial function**

The objective was to determine how regular exercise at either high (85-90%) or moderate (65-70%) intensity exercise affects VO_{2max}, cardiomyocyte contractile capacity and arterial endothelial function.

1.   High and moderate intensity exercise increased VO_{2max} 71% and 28%, respectively.

2.   High intensity exercise increased cardiomyocyte size 14% by proportional length and width growth, whereas moderate intensity increased cardiomyocyte length by 5% and cell width indicated growth. Ventricular weights and echocardiography indicated intensity-dependent biventricular hypertrophy.

3.   Cell fractional shortening increased 45% and 23% with high and moderate intensity exercise, respectively. Intensity-dependent resting bradycardia was indicated.

4.   Cardiomyocyte contraction rates increased 43% and 39%, whereas relaxation rates increased 20% and 10% with high and moderate intensity exercise, respectively. Rates of systolic and diastolic Ca^{2+} transient increase and decay paralleled contraction and relaxation rates.

5.   Diastolic Ca^{2+} levels and Ca^{2+} transient amplitudes were unaffected by exercise training, whereas Ca^{2+} sensitivity index increased 40% and 30% after high and moderate intensity exercise, respectively.

6.   Arterial endothelium-dependent relaxation improved with high and moderate intensity exercise; acetylcholine concentration evoking half-relaxation decreased 4.3-fold and 2.8-fold with high versus moderate intensity, but the difference between the exercise intensities was not statistically significant.

7.   Differential integrative effects were paralleled by intensity-dependent adaptations on cardiomyocyte hypertrophy and myocyte contractile function, but not so clearly with arterial endothelial function. Multivariate regression analysis showed that rates of systolic Ca^{2+} increase and diastolic cell relaxation corresponded best with VO_{2max}, although also degree of cell hypertrophy and contraction, and vasorelaxation correlated to VO_{2max} with univariate analysis.

PAPER IV   **Aerobic fitness is associated with cardiomyocyte contractile capacity and endothelial function in exercise training and detraining**

The objective was to determine the time-course of VO_{2max} and associated cellular changes after regular high intensity exercise was implemented and withdrawn (detraining).

Time-Course of Adaptations to Implementation of Exercise Training (2-13 Weeks)

1. VO_{2max} and cardiomyocyte size, fractional shortening, and rates of myocyte relengthening and Ca^{2+} decay improved steadily up to ~8-10 weeks.

2.   Multivariate regression analysis identified cardiomyocyte elongation and diastolic function as the main factors for determining VO_{2max}, although cell shortening and volume also correlated with VO_{2max} in univariate analysis.

Time-Course of Adaptations to Detraining (2-4 Weeks)

1.   About 50% of the exercise-gained increase in VO_{2max} vanished within 2 weeks of detraining, and plateaued 5% above sedentary controls after 3-4 weeks.

2.   Cardiomyocyte length and width increased 20-22% with exercise training. During detraining, cell width regressed completely within 2 weeks, whereas length remained 7% increased after 2 weeks and 5% after 4.

3.   Myocyte fractional shortening increased 30% with exercise, but vanished almost completely within 2 weeks of detraining. The training-induced increase in contraction-relaxation rates and corresponding Ca^{2+} transient rates regressed within 2-4 weeks.

4.   Diastolic Ca^{2+} levels and Ca^{2+} transient amplitudes were unaffected by exercise training and detraining, whereas Ca^{2+} sensitivity index increased with training, and regressed gradually within 4 weeks of detraining.

5.   Endothelial function improved after exercise training, and regressed completely within 2 weeks of detraining.

6.   Cardiomyocyte length and endothelial function appeared as the main cellular determinants for regressed VO_{2max} with detraining (multivariate regression analysis), whereas univariate analysis also showed that fractional shortening and rates of contraction-relaxation and Ca^{2+} transient correlated significantly with VO_{2max}.

DISCUSSION

The working hypothesis of the present thesis was that VO_{2max} is associated with cellular changes in the heart and in arteries. The magnitude of change depends on exercise intensity. Adaptations occur shortly after initiating regular exercise training, level off after ~2 months, and vanish almost completely within a month of detraining. Given that the level of VO_{2max} is closely related to clinical outcome, optimizing exercise programs is likely to yield significant health benefits.

Evaluation of Test Procedures for Oxygen Uptake

The procedures for determining VO_2 during treadmill running were found reproducible and valid both for rats and mice (I and II). To be strict, this validates measurements of submaximal VO_2 and not VO_{2max}. However, hallmarks of appropriate procedures for determining VO_{2max} are linear relationship between submaximal VO_2 and running speed, leveling off of VO_2 despite continued increase in running velocity (VO_{2max}), reproducible values from the same individual when duplicating tests with different inclinations, respiratory exchange ratios >1.0, linear heart rate response up to maximal levels (I), and clinical signs of exhaustion. These results are indicative of appropriate experimental procedures for determining VO_{2max} in rats and mice (I and II). Our methods resemble clinical procedures in healthy and diseased human individuals (181). Thus, the procedures were valid to determine relative exercise intensity and evaluate adaptations to regular exercise training.

Exercise Training Improves Maximal Oxygen Uptake and Cardiac Function

As expected, VO_{2max} and maximal aerobic running velocity increased with regular exercise training (I-IV). Improvements on VO_{2max} of 50-70% in rats and 30-50% in mice are among the largest reported, which probably reflects the close control of relative exercise intensity (for comparisons, see Discussion sections in papers I and II). Although several factors influence aerobic fitness, VO_{2max} is by far the most important (77,145). Nonetheless, regular exercise also improved work economy considerably, as VO_2 and respiratory exchange ratio during submaximal running decreased. VO_{2max} is closely related to cardiac function (59,194). Thus, improved cardiac output accounts for a large component of increased VO_{2max} (176,177), as

confirmed by increased oxygen pulse (I), which indicates larger stroke volume. Both diastolic and systolic function and hypertrophy of the heart account for higher cardiac output. Multiple regression analysis identified hypertrophy, diastolic function, and Ca^{2+} handling of the cardiomyocyte as important cellular mechanisms of increased VO_{2max}, whereas single univariate analysis also found that myocyte systolic function correlated well with VO_{2max} (III and IV).

Maximal Oxygen Uptake is Pliable to Different Exercise Intensities

High versus moderate exercise intensity during training sessions doubled VO_{2max}, even though moderate intensity induced a substantial increase (III). In fact, moderate exercise intensity increased VO_{2max} to a degree previously regarded as high (134). Recently, intensity-dependence of training-induced VO_{2max} has been confirmed in clinical trials of cardiovascular patients; high intensity exercise was superior for clinical outcome (see Introduction). This has important consequences since epidemiological data suggest that VO_{2max} is closely linked to health and survival (93,136). Surprisingly though, no studies have thoroughly examined the effects of different exercise intensities on VO_{2max} in healthy individuals during long-term exercise programs, although clues of intensity-dependence were provided early on also in healthy adults (39).

Time Course of Adaptations in Maximal Oxygen Uptake

Implementing regular high intensity exercise in previously inactive individuals imposes a rapid augmentation in VO_{2max} up to ~8 weeks, whereupon it leveled off (IV). This probably reflects that at one point, exercise training shifts to maintaining fitness, leaving little room for improvement unless higher doses of exercise are implemented (195).

The time course of regressed VO_{2max} is considerably shorter in rats than humans, as VO_{2max} perished almost completely to sedentary levels in less than 1 month (IV). Ten days of detraining in well-trained men did not reduce VO_{2max} (34), whereas 8 weeks detraining in previously active men and women only modestly decreased VO_{2max} (152). However, the initial level of fitness in the latter study was much lower than in paper IV.

Cardiomyocyte Hypertrophy with Training

Cardiomyocyte hypertrophy was shortly induced after initiating a regular exercise training program, and continued steadily for ~2 months, when hypertrophy plateaued (IV). The magnitude of cardiomyocyte hypertrophy was dependent upon the intensity of regular exercise, as high intensity exercise training induced a substantially more pronounced response than moderate intensity, 14% vs. 5% longer cells, respectively (III). Cell width also increased after high intensity exercise (III and IV), but was only indicated with moderate intensity (III). Thus, high intensity exercise provides a stronger hypertrophy stimulus than moderate intensity. Moreover, proportional cardiomyocyte length and width hypertrophy was also induced by high intensity exercise in mice.

Physiological hypertrophy is considered beneficial to improve the cardiomyocyte contractile capacity. It provides a cellular basis for larger end-diastolic dimensions, and confirms with the concept of athletes' heart, i.e. enlarged left and right ventricular masses and volumes, and increased wall thicknesses (58,146,150). Global hypertrophy was indicated by increased left and right ventricular weights (I-IV), and by echocardiography indices of enlarged left ventricular posterior wall and septum thickness (II, III). Exercise-induced cardiomyocyte hypertrophy corresponds to previous studies (8,129,187,188), including previous studies reporting increased heart weights in both endurance-trained rats (49) and mice (4,92,125). In fact, the time course of cardiomyocyte hypertrophy (IV) corresponds well to a study finding heart weights to plateau after ~10 weeks of regular exercise, following a continuous increase from initiation of exercise (33). In contrast, there is a lack of well-controlled studies on intensity-dependence of cardiac hypertrophy; in fact, most experimental studies do not control exercise intensity but rely on fixed treadmill speeds, voluntary wheel running, or swim training. This is probably because controlling exercise intensity accurately in terms of VO_2 requires more sophisticated and time-consuming protocols. Intensity-dependence of hypertrophy may explain why only top endurance athletes develop hypertrophied, athletic hearts, whereas less intense programs usually do not (146,147). Cardiac factors account for the ability to increase stroke volume linearly with exercise intensity up to near-exhaustion in well-trained endurance athletes (59,194), and not just up to ~50-60% of VO_{2max} as previously suggested (195). This is

probably why the magnitude of cardiomyocyte adaptation depends on exercise-intensity beyond moderate levels.

Hypertrophy Regression with Detraining

Regression of cardiomyocyte hypertrophy with inactivity following a period of regular exercise (IV) is consistent with previous studies of deconditioning after regular swim training in rats. After 9-10 weeks of daily unloaded swimming that increased heart weights similar to the present studies (I-IV), 80% of the gained heart weight was lost after 2 weeks of detraining (56), and 100% after 3 weeks (33). In fact, 60% of training-induced gain in heart weight was lost one week after stopping regular exercise (79). In the latter study, the training period lasted only 3 weeks, rendering the degree of hypertrophy uncertain. Finally, it was shown that both training- and detraining-induced changes in heart weight are repeatable and susceptible to successive exercise programs (78). Jointly, our data and the previous reports suggest that cardiomyocyte size is closely regulated according to changes in physiological needs.

Regression of cardiomyocyte size is probably the mechanism of myocardial hypertrophy regression in humans, although time course may be slower. Ten days of detraining did not induce any regression in well-trained athletes (34), whereas hypertrophy regression was evident after 3 weeks of detraining in athletes with similar endurance level (120). Thus, the rate of global cardiac regression in humans is probably considerably longer. About ~50% of former elite athletes experienced complete regression of left ventricular mass, whereas almost all maintained enlarged chamber volume during 13 years of follow-up after ceasing competitive sports (147). Given the participants history of exercise training, it is posible that continued leisure activity may have maintained a certain degree of hypertrophy.

Cardiomyocyte Contribution to Myocardial Changes

Cardiac myocytes seem to have an ample potential of adaptation to regular exercise training and inactivity. Although the present results (I-IV) did not quantify contributions of water and connective tissue, other studies have. The roles of connective tissue and collagen were minimal for regulation of cardiac weights during exercise training and detraining (78,79),

whereas myocardial hydration did not contribute at all (33). However, the latter study

indicated a minor role for non-myocyte protein components to changed heart weights. Thus,

the combined data show that cardiomyocyte size account for the major part of training-

induced cardiac hypertrophy and detraining-induced regression.

Mechanisms of Cardiomyocyte Size Adaptation

Physiologic hypertrophy has been associated with proportional increase in length and width of

the cardiomyocyte (83), but this has not been demonstrated experimentally until now (II-IV).

Other studies in our laboratory (I; 187,188) and elsewhere (129) have only reported increased

cell length after regular training. The reason for this discrepancy is presently unclear, but may

rely on the accuracy of measurements and number of cells studied. Proportional hypertrophy

requires generation of new sarcomeres in series and parallel. Longer cardiomyocytes do not

have longer sarcomeres (129), whereas tissue generation by differentiation, dedifferentiation,

or stem cell proliferation remains dubious, although such traces have been found in infarcted

hearts (12). Regression of cell length and width also occurs proportionally (IV).

Physiological and pathological hypertrophy is characterized by different molecular phenotypes

and gene expression profiles (85,86). The underlying molecular mechanisms of adding and

removing sarcomeres have not been determined, but several important mechanisms have

been identified. Physiologic growth appears to rely on initiation of the phosphoinositol-3-

kinase/AKT pathway to stimulate mTOR/p70 S6K signal transduction to increase rate of

protein translation in the ribosomal machinery (19,25,125). Other suggested targets involve

the Ca^{2+}-binding protein S100A1 (131) and the transcription factors GATA4 and GATA6

(102). Signal cascades normally associated with pathologic growth, such as atrial and brain

natriuretic peptides (ANP and BNP; 108) and reprogramming into a fetal gene program (83)

are usually depressed during physiological hypertrophy, although mildly increased mRNA

levels of cardiac ANP and BNP also have been reported with mice voluntary running (4).

Hypertrophy regression has been linked to depressed protein synthesis (56). Other

mechanisms could also contribute, e.g. increased rates of protein metabolism or degradation

to reduce redundant sarcomere mass.

Cardiomyocyte Contractility during Exercise Training and Detraining

Regular exercise training enhanced cardiomyocyte fractional shortening and rates of systolic contraction and diastolic relengthening (III and IV). The magnitude of improvement was larger with high intensity exercise than with moderate intensity (III). The adaptations increased steadily with regular exercise until a plateau was reached after ~2 months, whereas during detraining, adaptations subsided within 2-4 weeks (IV). No previous accounts exist of time course of cardiomyocyte contractile function after exercise training with different intensities or during detraining, but magnitude of improvements were in line with most data (e.g. 43,46,129, 187,188), but not with studies reporting no effect or decreased contractility (98,144,193). Different exercise training protocols and experimental conditions, e.g. ionic concentrations, pH, temperature, stimulation frequencies, and region of the heart from which cells were isolated, may explain some of the variation. Most studies did not stimulate cells up to physiological frequencies (7-10 Hz; e.g. 98); where our exercise-induced effects were most marked (III and IV). Together with hypertrophy, improved contractility effectuates a substantially greater contractile capacity of the cell, which contributes to greater systolic output and diastolic filling. Increased stroke volume is usually associated with resting bradycardia, as indicated in II and III.

Electrical versus Current Stimulation of Cardiomyocytes

Field stimulation does not completely resemble ordinary action potential-derived excitation-contraction coupling and should thus only cautiously be compared to patch-clamped cells stimulated with ion currents. For instance, field stimulation changes triggering mechanisms into dependence on L-type Ca^{2+} current and reverse mode NCX-current, instead of initiation by the Na^+ current, with the consequence that time to contraction is prolonged (24). In our hands, the extent of cell shortening with field stimulation has continuously been among the largest compared to other laboratories, with values regularly between ~15-25% at 7 Hz stimulation. Fractional shortening varies reportedly between ~3% and ~25%, in line with concordantly large variations in $[Ca^{2+}]_i$ amplitudes (15). Such differences probably result from experimental conditions, e.g. temperature, pH, concentrations of Ca^{2+} and other ions, stimulation frequencies, selection of cells, species and strains, and procedures for isolating

and culturing cardiomyocytes. These factors may also partially explain the controversy regarding a positive or negative force-frequency relationship (21,53). In our experimental conditions and procedures, we typically observed a positive staircase during 1-7 Hz stimulation, but negative >7 Hz (papers III and IV). This relationship is also maintained in heart failure cells (109). Furthermore, our approach studies intrinsic, unloaded contractile properties of the single cardiomyocyte, as mechanical influences by adjacent cells, tissues and impending in vivo load, as well as neurohormonal stimulation are removed.

Calcium Handling is Linked to Contraction-Relaxation Rates

The inter-dependency and the similar changes on $[Ca^{2+}]_i$ transients and contraction-relaxation velocities suggests that increase in rate of Ca^{2+} cycling explains faster shortening and relengthening of the trained cardiomyocytes (III and IV). Reduced rates of Ca^{2+} cycling with detraining also translate into slower contraction-relaxation rates (IV).

Altered Ca^{2+} handling and contractile capacity of the cardiomyocyte (III and IV) suggest that cellular adaptations contribute to improved global heart function in endurance-trained subjects, such as increased end-diastolic volume, stroke volume, and cardiac output (59,155, 162,194). Cell shortening has also been linked to myocardial contractility in heart failure (109). Regressed Ca^{2+} handling (IV) probably contributes to the detraining-induced reduction of stroke volume (32,120). Intensity-dependence of cardiac function has not been shown previously, but based upon paper III, we hypothesize that high intensity exercise is superior to moderate intensity to induce cardiac effects. Our results are of unloaded shortening with zero external work output, whereas in vivo loaded shortening includes external work due to tissue mass and blood pressure. However, single cell force- and power production also increase with regular exercise (43). Together, these results form a cellular basis for increased myocardial capability to perform external work, although effects of mechanical stress and neurohormonal stimulation are not accounted for (see Methods section for details).

Mechanisms of Altered Calcium Handling

Molecular changes that mechanistically explain the observed changes in Ca^{2+} handling or excitation-contraction coupling have not been determined, although clues are provided. More effective coupling of the L-type Ca^{2+} current to RyR2 Ca^{2+} release, but not more channels, has been shown with regular exercise (128,144). This suggests a mechanism for exercise-induced higher rate of systolic $[Ca^{2+}]_i$ transient increase (III and IV). Training-induced effects on isolated SR Ca^{2+} release *per se* have not been studied, but could potentially be interesting as the RyR2 complex is responsive to different stimuli (35,55,113,183). Faster $[Ca^{2+}]_i$ transients decay after exercise training (III and IV) seems to at least partly be explained by increased levels of SERCA2a, PLN and NCX (114,187,188). This improves Ca^{2+} re-sequestering and myocyte relaxation, and may secondarily improve contraction due to improved loading of the SR.

Calcium Sensitivity Accounts for Fractional Shortening

In rats, changed cardiomyocyte fractional shortening with exercise training or detraining could not be explained by systolic or diastolic levels of $[Ca^{2+}]_i$ or transient amplitude (III and IV). This suggests that the magnitude of cell shortening results from improved myofilament responsiveness to Ca^{2+}. Indeed, papers III and IV indicate that Ca^{2+} sensitivity improves with regular exercise training according to exercise intensity, and wanes with detraining. Adaptation of cardiac Ca^{2+} sensitivity to exercise training has previously been demonstrated thoroughly in rats (46) and has therefore been suggested as an important mechanism of intensity-dependency and time courses of increase and regression of cell fractional shortening (III and IV). Length-dependence of sarcomere Ca^{2+} sensitivity; stretched sarcomeres producing more force (44), implies that end-diastolic volume also may explain why myofilament Ca^{2+} responsiveness changes. These studies may also support the notion that Ca^{2+} sensitivity may be a compensatory mechanism to volume overload (148). Since the variation on Ca^{2+} sensitivity among different species is considerable (67,69,70,109,148,164) and no supportive data beyond rats exist (46, 188), the possibility remains that improved Ca^{2+} sensitivity is species-specific.

Mechanisms of Calcium Sensitivity

Likely mechanisms of changed Ca^{2+} sensitivity (III and IV) are found in the contractile machinery of the cell. The levels of atrial myosin light chain-1 in the heart (45), but also isoform shifting of troponin T (7), troponin I (100), and myosin heavy chains (137) have been associated with altered Ca^{2+} sensitivity. Regulation of pH may also affect Ca^{2+} sensitivity since protons may compete with Ca^{2+} and inhibit Ca^{2+}-binding to troponin C (141).

Molecular Coupling Between Hypertrophy and Contractility

Exercise training induces simultaneous adaptations on contractile function and size of the single cardiomyocyte (III and IV; see figure 2E paper IV). If the newly generated contractile machinery also contributes to improved contractility, then it suggests that intracellular regulatory mechanisms are activated, such as different protein kinases and phosphatases (e.g. 135). An interesting hypothesis is that the AKT pathway stimulates hypertrophy and facilitates Ca^{2+} cycling in concert (25).

Endothelium-Dependent Arterial Relaxation

Regular exercise training induced greater arterial endothelial function, but the effects were not significantly different between high and moderate exercise intensity (III), and vanished completely within days of detraining (less than 2 weeks; IV). Endothelium-mediated vasorelaxation measures the conductive ability of the artery (vasodilation), although endothelial function also includes mechanisms that protects against atherosclerosis (see Introduction). Exercise may therefore reverse endothelial dysfunction and exert anti-atherosclerotic effects on the endothelium. Thus, some effects of exercise training may be more marked in individuals with endothelial dysfunction.

Intensity-Dependence of Endothelium-Dependent Relaxation

Although endothelium-mediated vasorelaxation was indicated better after high intensity exercise training versus moderate, no statistical significance occurred between the exercise levels (III). No clear consensus has evolved on endothelial adaptations to exercise training in healthy individuals. Despite endothelium-dependent vasodilatation improving with moderate

intensity exercise (50% of VO_{2max}), it did not improve with high (75%) or low (25%) exercise intensities (61). Other reports show that endothelial function may improve (29,99,124), decrease (13) or remain unchanged (95,117,123) during regular exercise training. It was also reported that endurance-trained women do not have better endothelial-mediated vasodilatation than sedentary counterparts, but have structurally greater artery diameters (127). These discrepancies may be due to inadequate exercise programs, experimental procedures, selection of the specific artery to study (e.g. large conduit artery vs. smaller and more responsive arteries), initial status of the endothelium (normal vs. unhealthy), and inherent differences between human and animal biology.

In individuals with heart failure and cardiovascular disease, endothelial adaptation is associated with exercise-induced improvement of cardiovascular health and work capacity (3,71-73,97,106). This suggests that arterial adaptive potential may be larger when endothelial dysfunction is present.

Regression of Improved Endothelium-Dependent Relaxation
Regular exercise carried out for 10 weeks improved endothelial function substantially (III and IV). When training was stopped, the effects returned to normal levels within less than 2 weeks (IV). Given the rapid decline, one may assume that regular exercise is necessary to maintain, not just induce, improved endothelial function. The time course of regression appears longer in humans, as endothelial function returned to baseline ~2 months after ceasing regular exercise in patients with coronary artery disease and heart failure (117,180). How quickly regression progress in healthy humans is uncertain; whereas NO levels were maintained after 4 weeks of detraining, plasma levels of NO regress within 8 weeks (116).

Mechanisms of Endothelial Adaptation
Since dilatory responses to Na^+ nitroprusside and reaction to acetylcholine after eNOS inhibition with L-NAME were unaffected by training or detraining (III and IV), altered vasodilatory effects were probably caused by acetylcholine-mediated NO from the endothelium (see figure 2, Introduction). Besides increased eNOS enzyme activity (71),

regular exercise training also increases protein levels of eNOS if the tyrosine kinase c-Src is present (40). Also, increased shear stress and transmural pressure induces higher endothelial reactivity via AKT and vascular endothelial growth factor (VEGF; 1).

Although endothelial adaptation seemed to have a different time course than cardiomyocytes (III and IV), univariate correlation analysis established a weak association with VO$_{2max}$ (III), whereas multivariate regression indicated that regressed endothelial function is associated with VO$_{2max}$ (IV). However, further studies are required to investigate adaptations of the endothelium to exercise, especially in subjects with endothelial dysfunction.

Cellular Mechanisms of Maximal Oxygen Uptake

It takes a certain amount of flexibility and adaptability to respond to exercise training and withdrawal thereof. This thesis shows that changes in VO$_{2max}$ during training and detraining are closely associated with adaptation in cardiac myocytes. It should be noted that statistical modeling to assign values to cellular determinants cannot provide causal proofs but indices, and does not take into account other variables not measured or controlled for. Nonetheless, it provides important targets for more defining studies. Although the scope of this thesis was on oxygen delivery and identifies cardiomyocyte contractile capacity as a main determinant of aerobic fitness, other biological factors are also important for integrative function after training and detraining, e.g. capillarization, mitochondrial mass, and oxidative and metabolic enzymes (96,132).

General Considerations and Clinical Implications

The data reported here are obtained in healthy rats and mice with no history of cardiovascular disease, and provide thus insight into processes occurring under normal physiological conditions. Thus, it remains uncertain how varying degree of disease would affect the results. Genetic background may also affect the outcome. Both young and old individuals, as well as different sexes may react differently to the interventions. Papers I and II indicate that sex differences mainly would appear on the magnitude of adaptations, not the nature. It also adds credibility to translating results from animal to man, that quantitative rather than qualitative

differences seem to separate excitation-contraction coupling between the species (14,15). In cases were human samples are available, a large degree of similarity between experimental and clinical studies seem to appear (e.g. 66-73).

The studies were conducted with the intention to identify mechanisms and form a basis for studies of exercise training as a therapeutic approach in heart failure and related conditions. The first such studies in our laboratory show comparable, beneficial effects of training in heart failure or in animals at risk of developing cardiovascular disease (187,189), in line with other studies reporting beneficial effects of exercise training in heart failure (134). Therefore, we studied intensity-dependence with a clinical approach by changing exercise intensity without changing exercise time. This is not a strict control of the sheer effects of exercise intensity per se, as volume is not adjusted with moderate intensity exercise, but provides evidence that clinical and cellular effects increase if exercise intensity is increased *within a given period of time "set" for exercise*. As described in paper III, if one were to appropriately adjust exercise time to match volume between groups, the exercise time would approach critical lengths in clinical settings. Further experimental and clinical studies of exercise training as a therapeutic tool, partly based upon the present data, are currently underway and show potentially large effects of increasing exercise intensity in patients, even when amount of work is the same.

Given the importance of VO_{2max} for predicting cardiovascular health and all-cause mortality in healthy and cardiovascular patients (93,136), our results should have consequences for designing appropriate exercise programs and studies. Nevertheless, we recognize that low-to-moderate exercise (175), and very intense short-bout sprint training (190-193), may also be valuable in both health and disease.

CONCLUSIONS

1. The experimental procedures for determining VO_{2max} and running economy were valid and reproducible in both rats and mice during inclined treadmill exercise. Thus, the protocols could be used to accurately evaluate adaptations to regular exercise training, and to determine the relative intensity of treadmill running.

2. Regular intensity-controlled exercise training induces robust integrative and cellular adaptations in rat and mouse. VO_{2max} and running economy improve. Cardiomyocyte hypertrophy is induced; and contractile function, Ca^{2+} handling and Ca^{2+} sensitivity increase. Arterial endothelium-dependent vasoreactivity to acetylcholine improved.

3. Regular exercise at high versus moderate intensity induces about two-fold greater adaptations on VO_{2max} and cardiomyocyte dimensions, fractional shortening, Ca^{2+} sensitivity, and velocities of contraction-relaxation and Ca^{2+} transient cycles. In contrast, arterial endothelial function was less dependent upon exercise intensity.

4. After implementing regular high-intensity exercise training, VO_{2max} increases rapidly until a peak level is reached after ~8 weeks. Cardiomyocyte adaptations including hypertrophy response, fractional shortening, and rates of contraction-relaxation and Ca^{2+} transients follow a similar pattern. When regular training is stopped, regression occurs about twice as fast; most of the effects vanished completely within 2-4 weeks, except cell size and VO_{2max}, which remained slightly elevated after 4 weeks of detraining. In contrast, training-induced arterial endothelium-dependent relaxation regressed completely within 2 weeks of detraining.

5. Aerobic fitness and VO_{2max} are closely associated with cellular function and adaptations to regular training at high and moderate exercise intensities and detraining. Multivariate correlation analysis identified cardiomyocyte hypertrophy, rate of Ca^{2+} increase and decay, myocyte relaxation, and endothelial function as the main variables linked to VO_{2max}. Thus, cardiac cell and endothelium features are dynamic and show great plasticity to exercise stimuli.
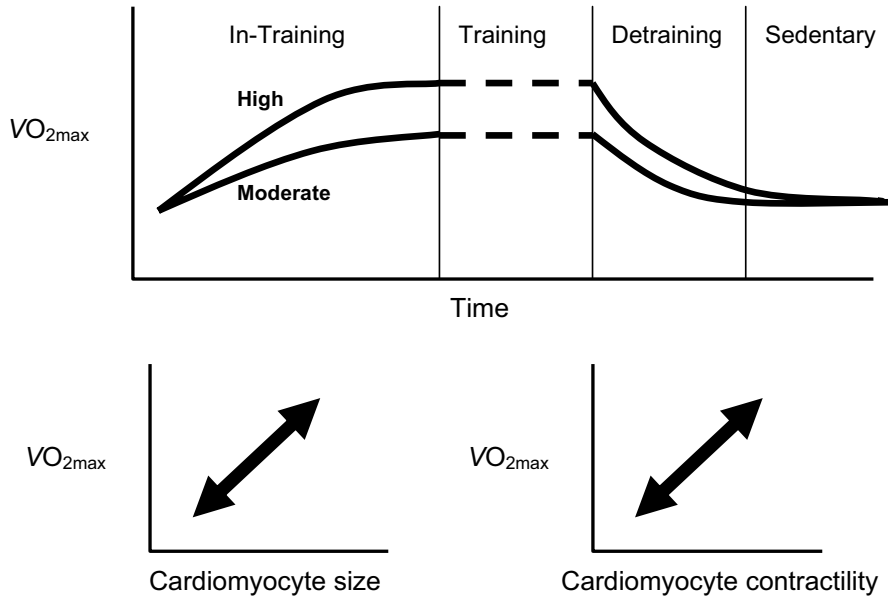
Figure 3. Summary of the main effects of exercise training at high or moderate intensity, and during detraining following regular exercise training.

The results indicate that exercise intensity is important for aerobic fitness and cellular adaptations that underlie health outcomes, and that cardiomyocyte contractile capacity emerges as an important determinant of VO_{2max}.

ERRATA

Paper IV

Page 2899, Figure 2 legend: references should read 6, 18.

Page 2899, last paragraph, line 4: "Figure 1" should read "Figure 2."

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