RESPIRATORY MUSCLE WORK AND INSPIRATORY MUSCLE TRAINING ON CYTOKINES, OXIDATIVE STRESS AND DIAPHRAGM FATIGUE IN YOUNGER AND OLDER POPULATIONS

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For Wesley

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ABSTRACT

Increased respiratory muscle work is encountered during strenuous whole-body exercise, and at rest in older adults and those with pulmonary limitations such as chronic obstructive pulmonary disease (COPD). When sufficiently strenuous it can result in diaphragmatic fatigue, increased blood lactate concentrations, and an alteration in respiratory muscle recruitment patterns. Increased respiratory muscle work also elevates cytokines interleukin-6 (IL-6) and interleukin-1β (IL-1β) within the respiratory muscles and systemically. There is mounting evidence that inflammation contributes significantly to the ageing process and age related diseases. Enhanced oxidative stress, glycogen depletion and diaphragmatic fatigue are all potential stimuli for this production. Whole-body exercise training can attenuate systemic inflammation and oxidative stress in younger adults during exercise, and in older adults who experience this at rest. An attenuation of muscle glycogen or increases in antioxidant enzymes may explain such reductions. Inspiratory muscle training (IMT) may also elicit similar adaptations in the inspiratory muscles, and thus also attenuate these markers. Accordingly, this thesis evaluated in younger adults whether: (I) the respiratory muscles contribute to exercise-induced increases in plasma cytokines and/or systemic oxidative stress measured by DNA damage to peripheral blood mononuclear cells (PBMC) during 1 h of steady-state cycling exercise (EX) and volitional hyperpnoea at rest which mimicked the breathing and respiratory muscle recruitment patterns achieved during EX (HYPEX) and heavy exercise (VH); (II) an increase in these inflammatory markers was related to diaphragmatic fatigue; (III) IMT attenuates these markers during EX, HYPEX and EX; and (IV) IMT changes an estimation of the maximum lactate steady-state and respiratory muscle recruitment patterns during the lactate minimum test. This thesis

also evaluated in older adults at rest whether: (V) IMT attenuates plasma cytokines and DNA damage to PBMC.

It was found in younger adults that: (I) plasma IL-6 concentrations increased during EX, HYPEX and VH and plasma IL-1β increased during VH. Plasma interleukin-1 receptor antagonist concentration and oxidative DNA damage to PBMC remained unchanged during VH; (II) the increase in IL-6 and IL-1β during VH was not related to the induction of diaphragmatic fatigue; (III) following IMT, plasma IL-6 concentrations were reduced by 33% during EX, 24% during VH, but were unchanged during HYPEX; and (V) following IMT, an estimation of the maximum lactate steady-state and respiratory muscle recruitment patterns remained unchanged during the lactate minimum test. It was found in older adults that: (IV) following IMT, nine plasma cytokines and DNA damage to PBMC remained unchanged. This thesis provides novel evidence that the respiratory muscles contribute to exercise-induced increases in plasma IL-6 and IL-1β concentration and that this increase is not related to diaphragmatic fatigue. IMT attenuates plasma IL-6 concentration during exercise, but not in a range of plasma cytokines in older adults at rest. It is attractive to speculate that the respiratory muscles contribute to the systemic inflammation observed in COPD patients and IMT may reduce the dysregulated cytokine response observed during exercise of COPD patients.

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ABBREVIATIONS

6MWT	six minute walking test
a–fv	arterial–femoral venous
ANOVA	analysis of variance
AMPK	adenosine monophospahte activated protein kinase
ATP	adenosine-5'-triphosphate
BAMPS	bilateral anterolateral magnetic phrenic nerve stimulation
BMI	body mass index
BP	blood pressure
BTPS	body temperature, pressure, saturated
COPD	chronic obstructive pulmonary disease
CO_2	carbon dioxide
CV	coefficient of variation
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme linked immunosorbant assay
EMG	electromyography
ENDO III	endonuclease III
EX	cycling exercise at the participants lactate minimum power
f_B	breathing frequency
f_C	cardiac frequency
FEV_1	forced expiratory volume in 1 s
F_ICO_2	fraction of inspired carbon dioxide
FPG	formamidopyrimidineglycosylase
FVC	forced vital capacity
GLUT4	glucose transporter type 4
gp130rα/IL-rβ	glycoprotein 130 homodimer and ligand binding chain
GM-CSF	granulocyte macrophage colony-stimulating factor
HYPEX	volitional hyperpnoea at rest whereby participants mimicked the breathing and respiratory muscle recruitment patterns achieved during cycling exercise at the participants lactate minimum power
IFN-γ	interferon-γ
IGF-1	insulin-like growth factor-1
IL-	interleukin
IL-1	interleukin-1
IL-1β	interleukin-1β
IL-1ra	interleukin-1 receptor antagonist
IL-2	interleukin-2
IL-6	interleukin-6
IL-6rα	interleukin-6 receptor α
IL-8	interleukin-8
IL-12 p70	interleukin-12 p70
IMT	inspiratory muscle training
IMT/EMT	combined inspiratory and expiratory muscle training
INC	incremental phase
IRL	inspiratory flow-resistive or pressure-threshold loading
ITL	incremental pressure-threshold loading
JNK/AP-1	c-Jun N-terminal kinase/activator protein-1

$[La^-]_B$	blood lactate concentrations
LEP	lactate elevation phase
MAX	termination of the lactate elevation phase
MEP	maximal static expiratory pressure
MIP	maximal static inspiratory pressure
MIP-1β	macrophage inflammatory protein-1β
MLSS	maximum lactate steady-state
mRNA	messenger ribonucleic acid
MRPD	maximum rate of inspiratory pressure development
MSNA	muscle sympathetic nerve activity
MVPA	moderate to vigorous physical activity
MVV	maximum voluntary ventilation
MVV_{10}	10 s maximal voluntary ventilation
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NO	nitric oxide
O_2	oxygen
OEP	optoelectronic plethysmography
OPQOL-35	Older People's Quality of Life Questionnaire
P38 MAPK	P38 mitogen activated protein kinase
P_{0max}	maximal inspiratory pressure at zero flow
P_{0opt}	optimal inspiratory pressure
PAL	physical activity levels
PASE	Physical Activity Scale for the Elderly
PASSIVE	passive rest
PAV	proportional assist ventilator
PBMC	peripheral blood mononuclear cells
PCO_2	partial pressure carbon dioxide
PCR	polymerase chain reaction
P_{di}	transdiaphragmatic pressure
P_{dipeak}	peak transdiaphragmatic pressure
P_{ditw}	transdiaphragmatic twitch pressure
P_e	oesophageal pressure
PEF	peak expiratory flow
P_{ETCO2}	partial pressure of end tidal carbon dioxide
P_{ETO2}	partial pressure of end tidal oxygen
P_g	gastric pressure
PI3-K	phosphatidylinositol 3-kinase
PIF	peak inspiratory flow
PLA	placebo
P_{MOUTH}	mouth pressure meter pressure
P_{STRAIN}	strain gauge pressure
PTP_{di}	diaphragm pressure-time product
PTP_e	inspiratory muscle pressure-time product
QoL	quality of life
REC	recovery phase
RER	respiratory exchange ratio
rh	recombinant human
RMT	respiratory muscle training
ROS	reactive oxygen species
RPE	rating of perceived exertion

RT-PCR	reverse transcription polymerase chain reaction
SD	standard deviation
SF-36	Short Form-36
SMD	standardised mean difference
SpO_2	estimated arterial oxygen saturation
STAT3	signal transducer and activator of transcription 3
sTNF-αr	soluble tumour necrosis factor-α receptor
STPD	standard temperature, pressure, dry
TBARS	thiobarbituric acid reactive substances
TIRE	Test of Incremental Respiratory Endurance
T_I/T_{TOT}	duty cycle
TNF-α	tumour necrosis factor-α
T_{di}	diaphragm thickness
$T_{di.CONT}$	diaphragm thickness during a Müeller manoeuvre from residual volume
$T_{di.FRC}$	diaphragm thickness at functional residual capacity
$T_{di.RV}$	diaphragm thickness at residual volume
$T_{di.TLC}$	diaphragm thickness at total lung capacity
$T_{di.TR}$	diaphragm thickening ratio
$\dot{V}CO_2$	carbon dioxide production
$\dot{V}_E$	minute ventilation
$\dot{V}_{Epeak}$	peak minute ventilation
$\dot{V}_{Imax}$	maximal inspiratory flow at zero pressure
$\dot{V}_{Iopt}$	optimal inspiratory flow
VH	volitional hyperpnoea at rest whereby participants mimicked the breathing and respiratory muscle recruitment patterns commensurate with heavy whole-body exercise
VIH	voluntary isocapnic hyperpnoea
$\dot{V}O_2$	oxygen uptake
$\dot{V}O_{2max}$	maximal oxygen uptake
$\dot{V}O_{2peak}$	peak oxygen uptake
V_T	tidal volume
$\dot{W}_{Imax}$	maximal inspiratory muscle power
$\dot{W}_{max}$	maximum power output
WOB	work of breathing
$\bar{\chi}$	mean

UNITS

%	percentage
°C	degree centigrade
cm	centimetre
d	day
g	gravity
h	hour
H_2O	water
Hz	hertz
kg	kilogram
L	litre
M	molar
mA	milliamp
mM	millimolar
MHz	megahertz
min	minute
mL	millilitre
mmHg	millimetre of mercury
pg	picogram
µg	microgram
revs	revolutions
s	second
V	volts
v	volume
wk	week
W	watt
yr	year

CHAPTER 1 – GENERAL INTRODUCTION

1.0 THE RESPIRATORY SYSTEM

The respiratory system is comprised of two interdependent components; the lung parenchyma which is responsible for gas exchange, and the respiratory muscles which act as the ventilatory pump. The primary function of the respiratory system is to preserve the partial pressure of arterial blood gases oxygen (O_2) and carbon dioxide (CO_2). During exercise there is an increased consumption of O_2 by the working muscles that must be maintained, and an increased CO_2 production that must be eliminated. Thus, the primary reason for increased ventilation during exercise is to increase alveolar ventilation in proportion to O_2 consumption and CO_2 production and maintain acid-base homeostasis. The respiratory system achieves this with remarkable precision and at minimal energy cost to the body through the co-ordination of the lung parenchyma, airways, respiratory control systems and respiratory muscles.

1.1 THE RESPIRATORY MUSCLES

The respiratory muscles are morphologically and functionally skeletal muscles. However, they only weigh ~960 g (Freedman et al. 1983), and represent only ~3% of total body mass (Robertson et al. 1977). The primary inspiratory muscles comprise the diaphragm, external intercostals, parasternal, sternomastoid and scalene muscles (Figure 1.1). The primary expiratory muscles comprise the internal intercostal, rectus abdominis, external and internal oblique and transverse abdominis muscles (Figure 1.1). The mechanical and kinematic analysis of the respiratory muscles during exercise has demonstrated that ventilation is made possible by highly coordinated actions of the respiratory muscles.

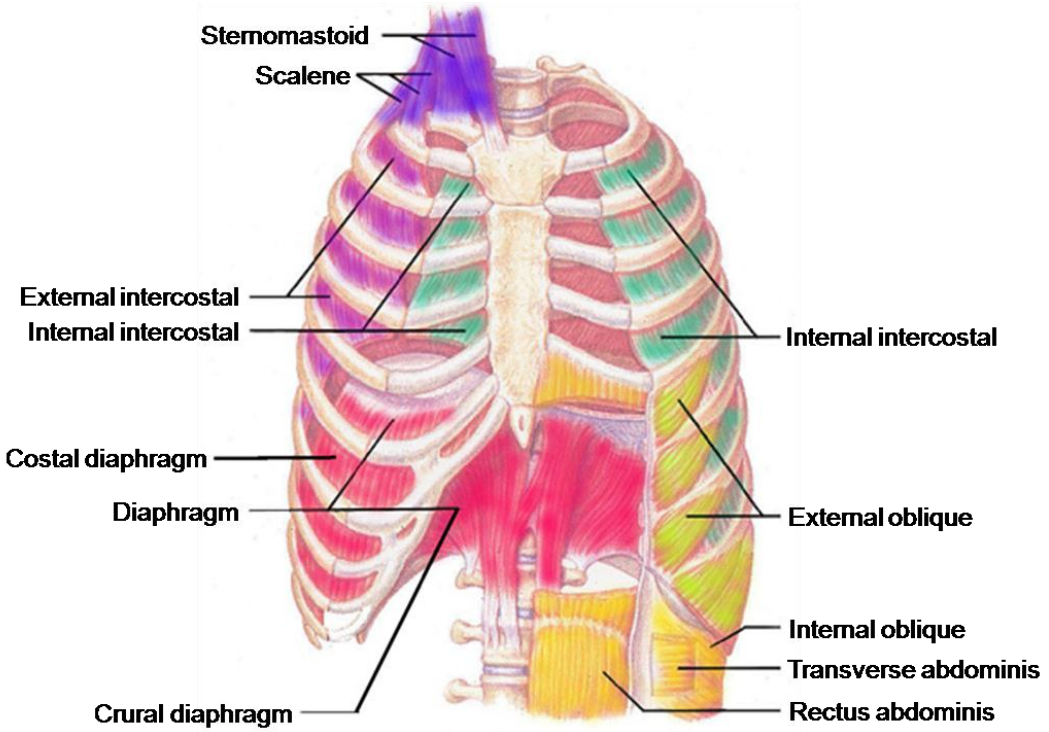


Figure 1.1 Schematic illustration of the anatomy of human respiratory muscles. Adapted from www.concept2.co.uk/training/breathing.php.

1.2 RESPIRATORY MECHANICS AND KINEMATICS DURING EXERCISE

1.2.1 RESPIRATORY MECHANICS AND KINEMATICS MEASUREMENT TECHNIQUES

The measurement of respiratory mechanics during exercise can be inferred from a balloon pressure catheter system. Thin walled balloon catheters are passed intranasally into participants with either two different balloon catheters, or two balloons sealed over a single catheter as shown in Figure 1.2. Pleural pressure is estimated from a catheter positioned in the oesophagus (oesophageal pressure; P_e). Abdominal pressure is estimated from a catheter positioned in the stomach (gastric pressure; P_g). The difference between pleural and abdominal pressure is termed transdiaphragmatic pressure (P_{di}).

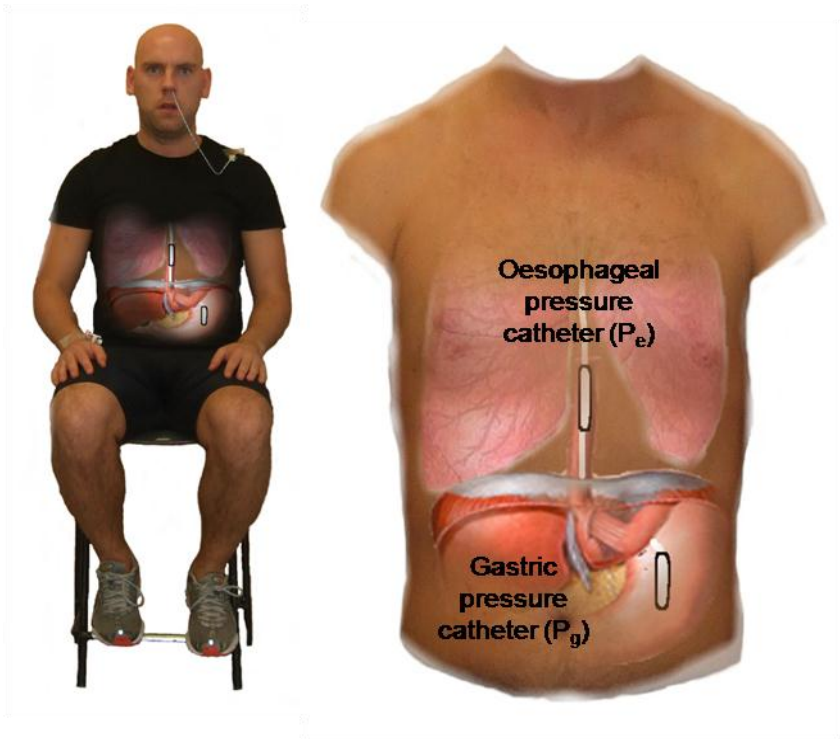


Figure 1.2 Balloon pressure catheter system with two balloons sealed over a single catheter. Note that internal organs are not to scale.

A limitation of the balloon catheter system is that it assumes that the chest wall moves with only two degrees of freedom. To overcome this limitation more recent studies have used a geometric optical reference system termed optoelectronic plethysmography (OEP; Figure 1.3). This system measures respiratory kinematics, and allows volume variations of the entire chest wall and its different compartments to be obtained breath by breath.

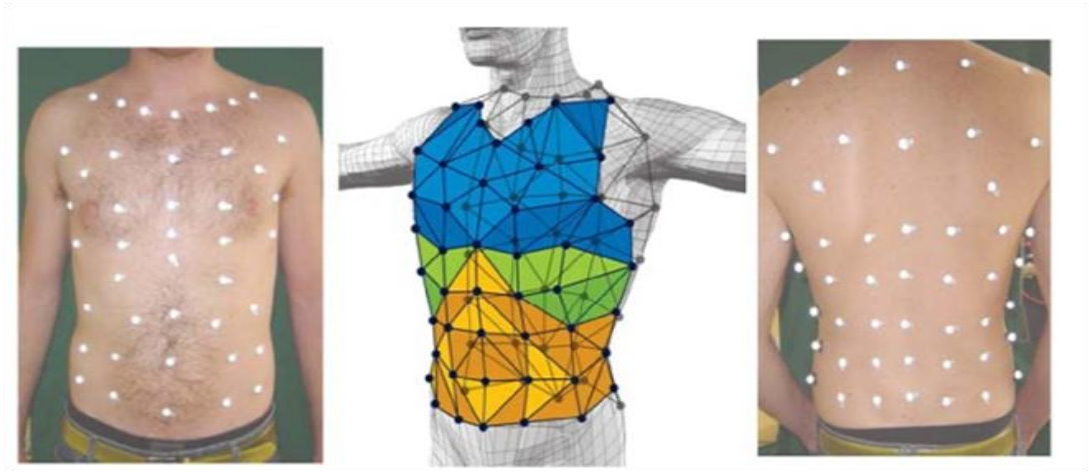


Figure 1.3 Optoelectronic plethysmography (OEP) uses a number of small reflective markers that are placed on the participant's trunk. A motion analysis system captures the three-dimensional coordinates of these markers and the enclosed volume is computed by connecting the points to form triangles. The chest wall can be modelled as being composed of three different compartments: pleural rib cage (blue), abdominal rib cage (green) and the abdomen (yellow). Adapted from Aliverti et al. (2009).

1.2.2 RESPIRATORY MECHANICS AND KINEMATICS DURING EXERCISE

Aliverti et al. (1997) have combined the kinematic measures from OEP with simultaneous measurement of pleural and abdominal pressure using a balloon pressure catheter system, enabling the measurement of the relative contributions of the chest wall muscles, the diaphragm and the abdominal muscles to the changes in compartmental volumes and pressures. Measurements were taken at rest and during sub-maximal cycling exercise at intensities of 0, 30, 50 and 70% maximum power output ($\dot{W}_{max}$). Their findings are summarised as follows: At rest, diaphragm shortening accounts for most of the increase in lung volume. At the onset of exercise (and with progressive intensities) end-inspiratory lung volume increases as a result of an increase in the volume of the pleural rib cage and to a lesser extent the volume of the abdomen. End-expiratory lung volume decreased as a result of a reduction in abdominal volume. As exercise progressed there was a gradual increase in the recruitment of rib cage and abdominal muscles. Throughout exercise there is a

gradual relaxation of the abdominal muscles during inspiration which causes a decrease in abdominal pressure and reduces P_{di}, whilst decreasing the expiratory action of the abdominal muscles on the volume of the abdomen. These findings suggest that during exercise the diaphragm acts primarily as a flow generator whereas the ribcage muscles generate the pressure to displace the abdomen and decrease end-inspiratory lung volume. The abdominal muscles generate the pressure to displace the abdomen and decrease end-expiratory lung volume. During inspiration the progressive increase in end-inspiratory lung volume acts to prevent ribcage distortion and simultaneously minimise P_{di}. This precise coordination of the respiratory muscles minimises the work of breathing (WOB) during exercise.

1.2.3 WORK OF BREATHING DURING EXERCISE

The WOB reflects the metabolic and/or energetic cost of the respiratory muscles to pulmonary ventilation and can be divided into two main categories: (i) elastic work - the energy required to alter the shape of the anatomical structures involved with breathing; and (ii) resistive work - the energy required to overcome the resistance to airflow in the airways. During exercise the respiratory muscles must perform both elastic and resistive work. Elastic work is performed to expand the lung and chest wall and overcome the elastic recoil force. Resistive work increases when the air entering the airways becomes more turbulent during high airflow rates.

The principle of 'minimal effort' is thought to govern the WOB during exercise. This principle has been supported by experimental data with mathematical modelling and demonstrates that the naturally occurring ventilatory response to exercise is associated with the minimal energy requirement from the respiratory muscles (Mead 1963; Otis et al. 1950; Yamashiro and Grodins 1973). Furthermore,

during exercise, ventilation occurs within a range of lung volumes that give the greatest total lung compliance (the change in pressure required to get a change in volume; ΔPressure / ΔVolume). The combination of the increase in tidal volume (V_T) and breathing frequency (f_B) occurs at a level that minimises dead space ventilation. Lastly, the respiratory muscles shorten at an optimal length so that force production is maximised.

Experimentally the WOB can be decreased using a proportional assist ventilator (PAV) or increased using inspiratory flow-resistive or pressure-threshold loading (IRL). The PAV partially unloads the inspiratory muscles by generating a positive pressure in proportion to inspiratory airflow and volume. IRL provides resistance to inspiratory airflow that requires participants to produce a negative inspiratory pressure sufficient to overcome the resistance. The resistances can be provided by a variable sized aperture or foil mesh screens for flow-resistive loading, or by using a spring loaded or weighted plunger device for pressure-threshold loading. When used during high-intensity exercise PAV and IRL (5 $cmH_2O \cdot L \cdot s^{-1}$ resistance) can reduce, or increase the WOB by ~40−60%, respectively (Harms et al. 1997; Harms et al. 1998). PAV and IRL can also reduce, or increase inspiratory muscle force output (integral of P_e multiplied by f_B and labelled the total inspiratory muscle pressure-time product) by ~30−80%, respectively (Babcock et al. 1995; Romer et al. 2006). However, using experimental approaches to manipulate the WOB has limitations. PAV or IRL possibly alters the recruitment of muscles involved with trunk stabilisation and locomotion (Calbet et al. 2007). Furthermore, unless a prescribed breathing pattern is imposed on participants during IRL, duty cycle (T_I/T_{TOT}; Clanton et al. 1985b), inspiratory flow rate (Clanton et al. 1985b;

McCool et al. 1986) and V_T (Clanton et al. 1990) increase to accommodate the mechanical load.

The WOB can be measured directly by mechanical work, or indirectly by measuring the O_2 cost of breathing. The mechanical WOB is estimated by simultaneously measuring the changes in intrathoracic pressures and the volume of the lungs throughout a respiratory cycle. In practice this is usually measured by integrating the inspiratory limb area of a P_e–V_T loop. The oxygen uptake ($\dot{V}O_2$) of the respiratory muscles can be measured by calculating the difference between whole-body $\dot{V}O_2$ at rest, or during exercise, and the $\dot{V}O_2$ during a volitional mimic at rest of the exercise hyperpnoea (volitional hyperpnoea). Isocapnia during volitional hyperpnoea is maintained at resting pressures by supplementing CO_2 into the inspiratory circuit. However, it is important to note that to have a valid estimate of respiratory muscle $\dot{V}O_2$ using volitional hyperpnoea the WOB must be rigorously controlled. When the WOB is not controlled, and the participant self-selects their own breathing and respiratory muscle recruitment patterns; respiratory muscle $\dot{V}O_2$ can increase by ~25% due to tachypnoea, the recruitment of less efficient respiratory muscles, and an increase in end-expiratory lung volume (Coast et al. 1993).

Using this rigorous experimental approach Aaron and colleagues (Aaron et al. 1992a; Aaron et al. 1992b) instructed participants to mimic at rest the breathing pattern (f_B) and WOB (end expiratory lung volume and P_e–V_T loop) associated with heavy exercise hyperpnoea. The difference between $\dot{V}O_2$ during volitional hyperpnoea, and $\dot{V}O_2$ at rest was used to calculate respiratory muscle $\dot{V}O_2$. As minute ventilation ($\dot{V}_E$) increased from 75 to 125 $L \cdot min^{-1}$ respiratory muscle $\dot{V}O_2$ increased from 1.8 to 2.1 mL O_2 per $L \cdot min^{-1}$ increase in $\dot{V}_E$. As a fraction of whole-body $\dot{V}O_2$ respiratory muscle $\dot{V}O_2$ in untrained adults was 3–5% for moderate intensity (75% of

maximal oxygen uptake; $\dot{V}O_{2max}$) exercise and 8−10% at maximal exercise. Respiratory muscle $\dot{V}O_2$ was higher (13−16%) in trained participants that had a $\dot{V}O_{2max}$ of >60 $mL \cdot kg^{-1}min^{-1}$. Regression analyses have shown that the WOB and respiratory muscle $\dot{V}O_2$ both increase linearly during maximal incremental exercise until the limit of volitional tolerance (Figure 1.4). This increase in respiratory muscle $\dot{V}O_2$ during exercise must be delivered with an appropriate increase in respiratory muscle blood flow.

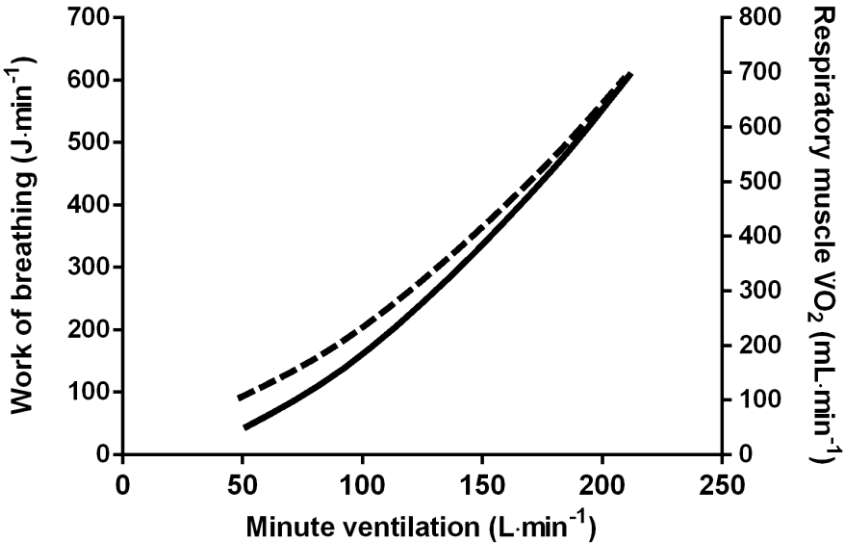


Figure 1.4 The estimated relationship between exercise ventilation and the work of breathing (solid line) and respiratory muscle oxygen uptake ($\dot{V}O_2$) (dotted line). Work of breathing estimation based on regression equation from Aaron et al. (1992a) where the work of breathing = -80.041 + 1.459 (minute ventilation) + 0.011 (minute ventilation) 2. Respiratory muscle $\dot{V}O_2$ based on a regression equation from Aaron et al. (1992b), where respiratory muscle $\dot{V}O_2$ = 0.081 + 0.001 (exercise work of breathing - resting work of breathing). Adapted from Sheel and Romer (2012).

1.3 RESPIRATORY MUSCLE BLOOD FLOW DURING EXERCISE

The quantification of respiratory muscle blood flow in exercising humans is difficult because of the complex anatomical arrangement and large vascular network that the respiratory muscles have. Therefore, most information regarding respiratory muscle blood flow during exercise has been derived from animal investigations

(Manohar 1986a; Manohar 1986b; Manohar 1988; Manohar 1990; Manohar and Hassan 1991). For example, diaphragmatic blood flow in ponies (measured directly via microsphere distribution) increased from 11.5 ± 2.8 $mL \cdot min^{-1} \cdot 100g$ tissue at rest to 265 ± 35.9 $mL \cdot min^{-1} \cdot 100g$ tissue at maximal exercise which was greater than all other respiratory and non-respiratory skeletal muscles. The studies from exercising ponies indicate that at maximal exercise the respiratory muscles receive ~15% of the total cardiac output with 8−9% and 5−6% directed towards the inspiratory and expiratory muscles, respectively. These values reasonably agree with measurements of respiratory muscle blood flow in a variety of species (Laughlin et al. 1989; Manohar 1986a; Nattie 1983) and with available estimates of respiratory muscle blood flow in humans (Harms et al. 1997; Harms et al. 1998).

Harms and colleagues (Harms et al. 1997; Harms et al. 1998) estimated respiratory muscle blood flow by simultaneously measuring cardiac output (Fick method) and leg blood flow (thermodilution) during several short duration (2.5 to 3.0 min), high-intensity cycling exercise tests in which the WOB was either reduced with a PAV or increased with IRL. When the WOB was reduced or increased there was a corresponding increase or decrease in leg blood flow. Cardiac output and stroke volume were decreased with the reduced WOB and remained unchanged with the increased WOB. Based on these findings Harms et al. (1998) estimated the distribution of total cardiac output available during maximal cycling exercise to be ~77% directed to the locomotor muscles, 14−16% to the respiratory muscles and 7−9% to other metabolically active tissues (Figure 1.5). However, the use of the PAV to unload the inspiratory muscles can reduce the negativity of intrathoracic pressure at end-inspiration, so that changes in blood flow can be partly attributed to reductions in cardiac output and stroke volume (Harms et al. 1997; Harms et al. 1998).

Interestingly, using the same experimental design, but a lower intensity (50 and 75% $\dot{V}O_{2max}$) and longer duration (15 to 20 min) exercise it was shown that there was no change in leg blood flow suggesting that there is a threshold above which this effect occurs (Wetter et al. 1999).

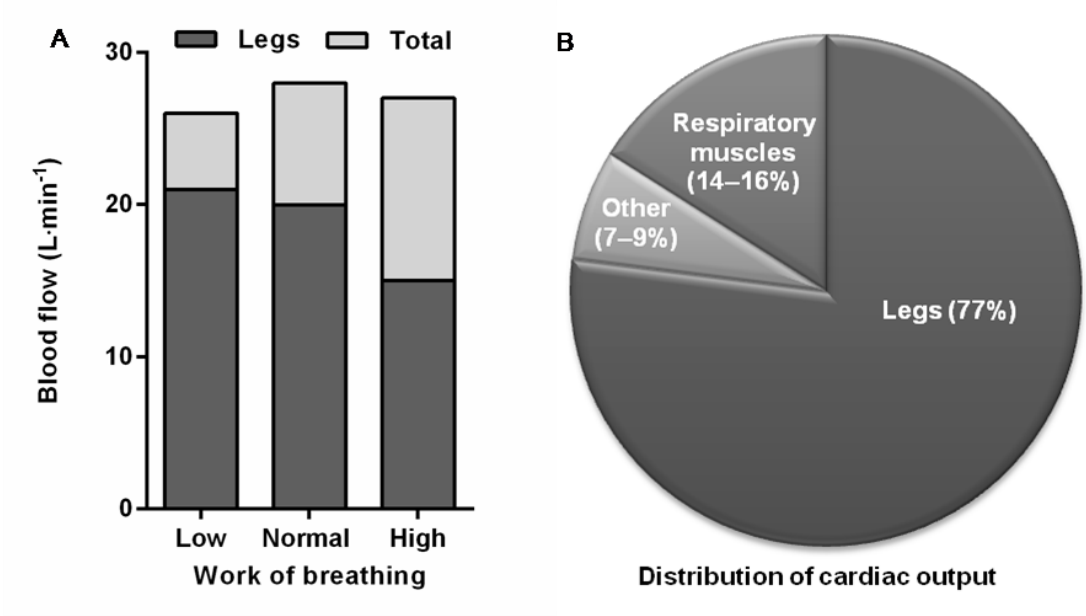


Figure 1.5 Estimation of average changes during maximum cycling exercise in the total cardiac output available (total blood flow; A) and distribution of cardiac output (B) based on research where the work of breathing was either reduced via a proportional assist ventilator (low), normal, or increased via inspiratory flow-resistive loading (high). Data from Harms et al. (1997 and 1998). Adapted from Dempsey (2012).

Recent studies have shown that there is the potential to directly measure respiratory muscle blood flow in exercising humans. Respiratory muscle blood flow has been estimated using the light-absorbing tracer dye indocyanine green in combination with near-infrared spectroscopy of the intercostal muscles during exercise (Habazettl et al. 2010; Henderson et al. 2012; Vogiatzis et al. 2008; Vogiatzis et al. 2009) and volitional hyperpnoea at rest (Guenette et al. 2008; Guenette et al. 2011). Using this technique it has been shown that respiratory muscle

blood flow (estimated from the seventh intercostal space) progressively increases during elevations of $\dot{V}_E$ up to 120 $L \cdot min^{-1}$, and there were strong positive correlations between blood flow, cardiac output, the WOB and P_{di} (Guenette et al. 2008). However the technique is currently limited as the optode positioned over the seventh intercostal space will measure all of the blood flow in view, and it is impossible to separate out the relative contributions from other muscles such as the diaphragm. It has been shown that the demands placed on the respiratory muscles during an increased WOB are substantial requiring, a considerable metabolic cost, and appropriate increase in respiratory muscle blood flow. This can occur due to the advantageous morphology of the respiratory muscles including efficient fibre types, high levels of enzyme activities, a large mitochondrial density, and a substantial vascular supply.

1.4 RESPIRATORY MUSCLE MORPHOLOGY

1.4.1 RESPIRATORY MUSCLE FIBRE TYPES

Muscle fibre type distribution (performed histochemically using myofibrillar ATPase staining) has shown that male human cadavers (reported to be previously healthy) have ~60% type I muscle fibres in the internal and external intercostals and 49% in the diaphragm (Figure 1.6; Mizuno and Secher 1989). Type II distribution was similar between the internal intercostals and the diaphragm (~30%), but only 17% in the external intercostals. In contrast, type IIx fibre type was similar between the external intercostals and the diaphragm (~25%), and only 1% in the internal intercostals. This finding is in agreement with muscle fibre type distribution in diaphragm biopsies taken from healthy humans using myofibrillar ATPase staining (Sanchez et al. 1982; Sanchez et al. 1985) and expression of myosin heavy chain

isoforms (Levine et al. 1997; Nguyen et al. 2000). Interestingly, the distribution of fibre types in the diaphragm is remarkably similar to that of the vastus lateralis (Figure 1.6) suggesting that the superior oxidative capacity of the diaphragm is not explained by fibre type distribution, but rather its enzyme activities, mitochondrial density and vascular supply. These additional morphological adaptations are not present in the accessory inspiratory muscles.

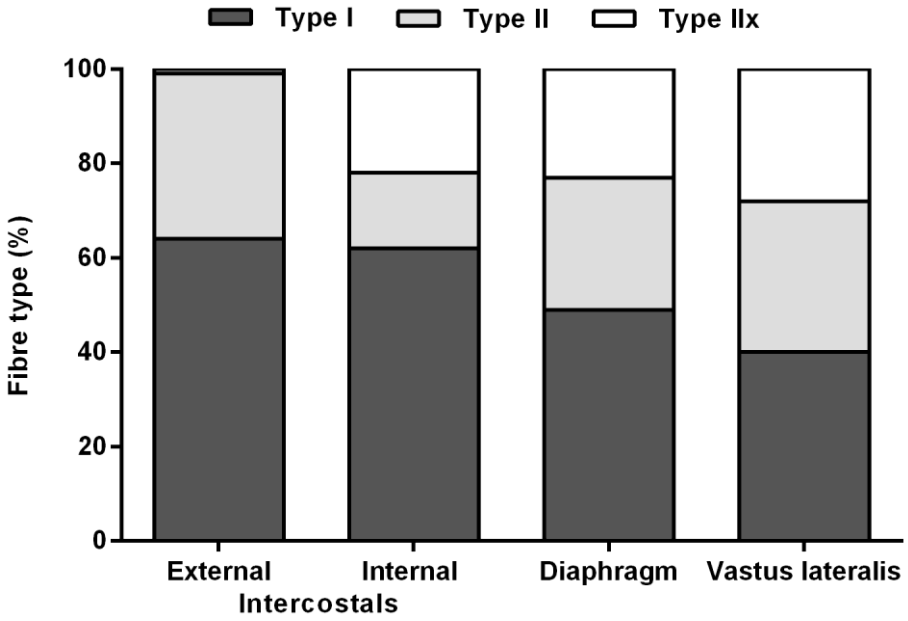


Figure 1.6 Relative distribution of slow twitch (type I), fast twitch a (type II), and fast twitch x (type IIx) muscle fibres in the external and internal intercostals, the costal diaphragm and the vastus lateralis taken from 8 male cadavers (previously healthy) who died aged 17 to 51 years (average 31 years). Adapted from Mizuno and Secher (1989).

1.4.2 RESPIRATORY MUSCLE ENZYME ACTIVITY, MITOCHONDRIAL DENSITY AND VASCULAR SUPPLY

Although measurements of respiratory muscle enzyme activities have been made in human cadavers (Mizuno et al. 1985); these are often compromised by the varied and long time periods between death and tissue removal (Mizuno 1991; Powers et al. 1997). There are, however, several reports that have shown that the

activity of oxidative (i.e., Krebs cycle) enzymes in healthy sedentary rats are ~2 times greater in the diaphragm compared with a locomotor muscle possessing similar fibre types (Powers et al. 1994a; Powers et al. 1994b; Sexton and Poole 1995). For example, Sexton and Poole (1995) have spectrophotometrically determined that resting citrate synthase (rate limiting enzyme in Krebs cycle) activity is higher in all parts of the diaphragm compared to the hind limb muscles of rats (Figure 1.7). Nevertheless, these large differences in oxidative capacity between the diaphragm and locomotor muscles could be explained by the activity of these muscles. The locomotor muscles of sedentary (caged) rats undergo minimal amounts of activity compared to the chronically active diaphragm. The reason for the greater oxidative activity could be due to the metabolically plastic nature of skeletal muscles (Powers et al. 1997).

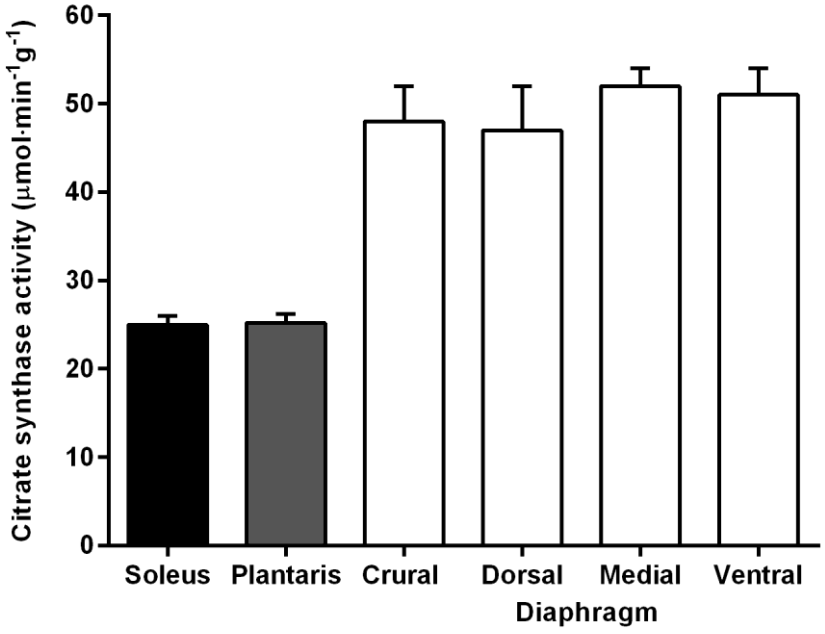


Figure 1.7 Citrate synthase activity of the hind limb muscles (soleus and plantaris) and regional parts of the diaphragm in rats at rest. Values are mean ± SE from Sexton and Poole (1995). Adapted from Poole et al. (1997).

The mitochondrial density of the respiratory muscles has only been examined in animals. In these, it has been observed using electron microscopy that the mitochondrial density in the diaphragm is ~2−3 times greater than that found in the hind limbs of both active and large (Hoppeler et al. 1981), and sedentary and small (Gamboa and Andrade 2010) mammals.

The vascular supply of the respiratory muscles is extensive and more closely reflects the vascular supply of the brain than other skeletal muscles (Comtois et al. 1987). Unlike locomotor skeletal muscles which have a single arterial supply; the diaphragm has multiple including the superior and inferior phrenic arteries, intercostal artery and internal mammary artery (Comtois et al. 1987). This multiple arterial supply provides a protective arrangement from ischemia. The analysis of human cadavers by Mizuno and Secher (1989) that is described above has also shown that, although the fibre type distribution of the diaphragm and vastus lateralis are similar, the number of capillaries per muscle fibre is higher in the diaphragm. The area and circumference of diaphragm muscle fibres is also smaller than all other skeletal muscles which minimises diffusion distance. The capillary bed of the diaphragm is also ~2−3 times greater than that found in other skeletal muscles (Hoppeler et al. 1981). In spite of these morphologically advantageous features, it is surprising that under certain conditions, the respiratory muscles (including the diaphragm) can still fatigue during exercise (exercise-induced diaphragm fatigue) and during strenuous contractions at rest through resistive loading or volitional hyperpnoea (breathing-induced diaphragm fatigue).

1.5 RESPIRATORY MUSCLE FATIGUE

Respiratory muscle fatigue is defined as "a condition in which there is a loss in the capacity for developing force and/or velocity of a muscle, resulting from muscle activity under load and which is reversible by rest" (NHLBI 1990). Respiratory muscle fatigue is evident from a reduced force output relative to the baseline value (Romer and Polkey 2008).

1.5.1 SITES OF RESPIRATORY MUSCLE FATIGUE

Central fatigue is a reduction in voluntary force output due to a decrease in the motor output from the brain to the motor neuron pool (Gandevia 2001). Central fatigue of the inspiratory muscles has been observed after IRL using the "twitch interpolation" technique where supramaximal stimulation is superimposed upon a maximal inspiratory effort (Müller Manoeuvre) (Bellemare and Bigland-Ritchie 1987; Gandevia and McKenzie 1985; McKenzie et al. 1992). A different method is to deliver a motor-evoked potential by the use of transcranial electrical (Gandevia and Rothwell 1987) or magnetic (Similowski et al. 1996) stimulation of the motor cortex. A decrease in the amplitude of the motor evoked potential from transcranial stimulation indicates a reduced excitability of the motor cortex due to supraspinal mechanisms. Transcranial stimulation has been used to show central diaphragmatic fatigue after whole-body exercise (Dayer et al. 2007; Verin et al. 2004). However, the functional consequences of a decrease in the motor-evoked potential remain unknown.

Peripheral fatigue is a reduction in force output due to processes distal to the neuromuscular junction (Fitts 1994). Peripheral fatigue can be categorised into high frequency and low frequency fatigue. High frequency fatigue results in depression of

the force output after high frequency stimulation (~50−100 Hz). In contrast, low frequency fatigue results in depression of the force output after low frequency stimulation (e.g., 1−30 Hz). It is believed that high frequency fatigue indicates interference in the propagation of the action potential along, or throughout, the t-tubule system whereas low frequency fatigue indicates impairment of excitation-contraction coupling (Jones 1996). Results from resistive loading and volitional hyperpnoea studies have reported that high frequency fatigue recovers quickly, after 10−20 min, whereas low frequency fatigue may require hours to fully recover (Aubier et al. 1981; Bai et al. 1984; Moxham et al. 1981). The following sections will focus only on peripheral fatigue.

1.5.2 RESPIRATORY MUSCLE FATIGUE MEASUREMENT TECHNIQUES

Peripheral respiratory muscle fatigue can be assessed with non-invasive and volitional measurements using maximal static inspiratory (MIP) or expiratory (MEP) pressures, maximal P_{di} or P_e and maximal sniff or cough pressure. These measurements have demonstrated respiratory muscle fatigue after high-intensity (Bye et al. 1984; Coast et al. 1990; Coast et al. 1999; Griffiths and McConnell 2007; Lomax and McConnell 2003; Romer et al. 2002c; Volianitis et al. 2001) and strenuous and prolonged endurance exercise (Chevrolet et al. 1993; Cordain et al. 1994; Hill et al. 1991; Loke et al. 1982; Ross et al. 2008) in a range of modalities. The major limitations of these techniques are that they are effort dependent and can be influenced by task learning or central drive (ATS/ERS 2002).

Respiratory muscle fatigue can also be assessed by analysing the frequency domain of electromyography (EMG) signals recorded using skin-surface electrodes positioned over the muscle of interest, or an electrode catheter passed intranasally

into the participant and positioned in the lower oesophagus for measurements of the crural diaphragm (ATS/ERS 2002; Luo et al. 2008). The rationale for frequency domain analysis is that fatigue can shift the power spectrum of the EMG to lower frequencies (Gross et al. 1979). Shifts in the EMG power spectrum have been reported after resistive loading (Bower et al. 1984; Fitting et al. 1988; Pardy and Bye 1985) and heavy whole-body exercise (Aldrich et al. 1983). However, the validity of EMG power spectrum measurements remains controversial. Neural or sarcolemmal events may be responsible for power spectral shifts with fatigue rather than processes involving excitation-contraction coupling (Sieck and Fournier 1990). Therefore, there is a poor correlation between EMG spectral shifts and mechanical indices of fatigue (Moxham et al. 1982). Technical complications such as electrode position, signal-to-noise ratio and recruitment of muscles that potentially contribute to crosstalk can also complicate measurement of the EMG power spectrum (ATS/ERS 2002; Luo et al. 2008).

With the methodological limitations of volitional measurements and EMG power spectrum described above; studies using these techniques to demonstrate respiratory muscle fatigue will not be discussed in this thesis. Arguably, the most valid method of assessing respiratory muscle fatigue is to estimate the force output of the muscle in question by measuring the intrathoracic pressure response to motor nerve stimulation.

Motor nerve stimulation is an objective, non-volitional measurement and can therefore measure the particular muscle in isolation and independent of effort. Electrical or magnetic devices provide stimulation and the force output is measured with a balloon pressure catheter system (Figure 1.2). For the diaphragm the phrenic nerves are stimulated, and the P_{di} response is measured (Figure 1.8). For the

abdominal muscles the abdominal wall or thoracic nerves are stimulated, and the P_g response is measured. The measurement of changes in the pressure-frequency response of the muscle can gain an insight into the underlying mechanisms of fatigue. The additional use of an EMG system allows the measurement of the compound muscle action potential evoked during stimulation (i.e., M-wave) to detect changes in neuromuscular transmission (Aldrich 1987).

To gain a valid measure of respiratory muscle fatigue using nerve stimulation several potential sources of error must be controlled; including abdominal compliance (Koulouris et al. 1989; Man et al. 2002), postactivation potentiation (Mador et al. 1994; Wragg et al. 1994), isovolumic conditions (Hubmayr et al. 1989; Smith and Bellemare 1987) and supramaximal stimulation (Davies and White 1982; Taylor et al. 1996).

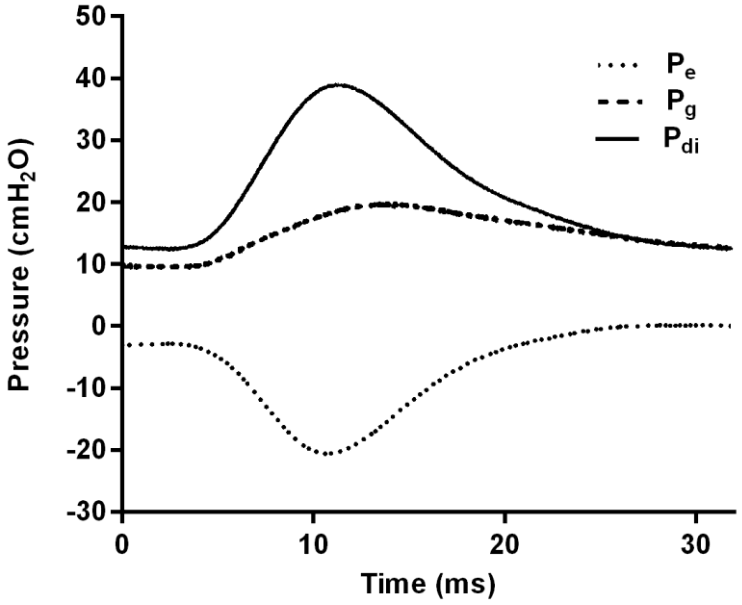


Figure 1.8 Oesophageal (P_e), gastric (P_g) and transdiaphragmatic pressure (P_{di}) response to single frequency supramaximal bilateral phrenic nerve stimulation from an anterolateral approach.

Whilst supramaximal stimulation is easy to achieve with electrical stimulation it is painful for participants. Thus, magnetic stimulation techniques with coils are more commonly used. For peripheral fatigue of the abdominal muscles; magnetic stimulation is undertaken by placing a circular coil over the thoracic nerve roots (Kyroussis et al. 1996). This technique has demonstrated exercise-induced abdominal fatigue (Taylor et al. 2006; Taylor and Romer 2008; Verges et al. 2007). For peripheral fatigue of the diaphragm; one method of magnetic stimulation of the phrenic nerves can be undertaken by placing a circular coil posteriorly over the midline between the fifth and seventh cervical vertebrae (Laghi et al. 1996). However, it has proved difficult to consistently achieve supramaximal stimulation with this, or with a similar anterior method (Mador et al. 1996; Polkey et al. 2000). The other method is to stimulate the phrenic nerves bilaterally from an anterolateral approach (BAMPS; Figure 1.9) which has been shown to be a reliable and valid nerve stimulation technique (Mills et al. 1996).

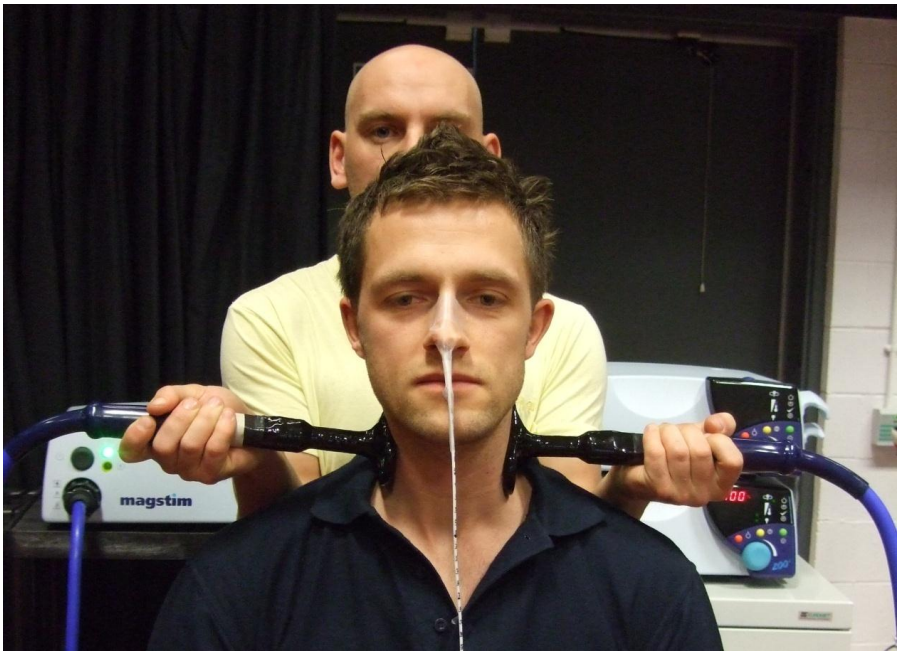


Figure 1.9 Bilateral anterolateral magnetic phrenic nerve stimulation (BAMPS) in conjunction with a balloon pressure catheter system.

1.5.3 EXERCISE-INDUCED DIAPHRAGM FATIGUE

The use of supramaximal bilateral phrenic nerve stimulation has demonstrated exercise-induced diaphragm fatigue at low (Babcock et al. 1995; Babcock et al. 1995; Babcock et al. 1996; Babcock et al. 2002; Johnson et al. 1993; Mador et al. 1993) and high frequencies (Babcock et al. 1998). Reductions of 15–30% in P_{di} have been observed within 10 min after short-duration exercise at intensities greater than 80–85% $\dot{V}O_{2max}$, and P_{di} does not return to baseline values until 1–2 h post exercise (Babcock et al. 1995; Babcock et al. 1995; Babcock et al. 1996; Babcock et al. 1998; Babcock et al. 2002; Johnson et al. 1993; Mador et al. 1993). For example, Johnson et al. (1993) reported diaphragm fatigue after trained ($\dot{V}O_{2max}$: 61 ± 4 $mL \cdot kg^{-1}min^{-1}$) participants undertook treadmill running or cycling exercise at 85 and 95% $\dot{V}O_{2max}$ for 31 ± 8 and 14 ± 3 min, respectively. There was a significant reduction in the P_{di} response to supramaximal electrical bilateral phrenic nerve single twitch (1 Hz) and tetanic stimulation (10 and 20 Hz) undertaken at a range of lung volumes from total lung capacity to residual volume. P_{di} partially recovered by 30 min and was still depressed at ~70 min (Figure 1.10).

It has been demonstrated that short-duration maximal incremental exercise until the limit of volitional tolerance does not alter the P_{di} response to supramaximal nerve stimulation (Romer et al. 2007) indicating that this type of exercise does not result in diaphragm fatigue. This can probably be explained by the short duration of exercise during which the cumulative work history of the diaphragm is not great enough to reach fatiguing levels (Romer et al. 2007). This observation suggests that both exercise intensity and duration play a part in exercise-induced diaphragm fatigue. To the author's knowledge no studies have used nerve stimulation

techniques to examine whether the diaphragm fatigues after longer duration constant load exercise with an initial intensity lower than 80−85% $\dot{V}O_{2max}$.

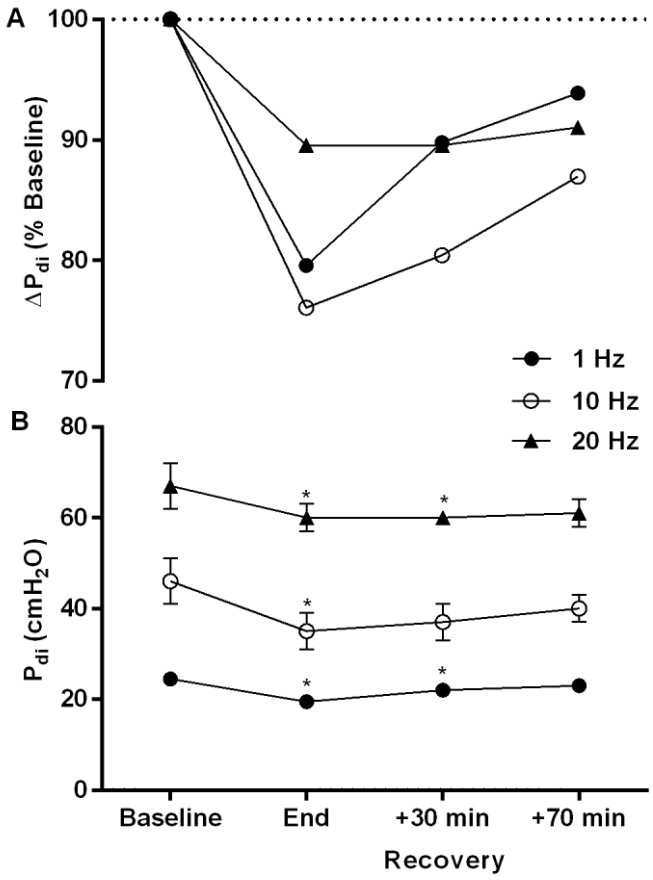


Figure 1.10 Transdiaphragmatic pressure (P_{di}) response to supramaximal electrical bilateral phrenic nerve single frequency (1 Hz) and tetanic stimulation (10 and 20 Hz) at functional residual capacity before and in recovery from running or cycling exercise at 95% maximal oxygen uptake shown as a percentage of baseline values (A) and absolute values (B). Significant difference (from baseline * $P < 0.05$). Values are mean ± SE for bottom panel. Adapted from Johnson et al. (1993).

Recently several reports have suggested that the diaphragm fatigues immediately after rather than during exercise (Kabitz et al. 2007; Kabitz et al. 2007; Kabitz et al. 2008a; Kabitz et al. 2008b; Kabitz et al. 2010). Using BAMPS it was shown that peak to peak amplitude of transdiaphragmatic (single frequency) twitch pressure (P_{ditw}) progressively increases during cycling exercise (~85% $\dot{V}O_{2max}$), but

fell immediately after the end of exercise. The authors' suggested that the increase in P_{ditw} during exercise was due to a rise in intracellular acidosis and respiratory drive which acts to increase diaphragm strength. However, these findings may be explained by methodological limitations. Most importantly, the studies did not correct for changes in lung volume during exercise. End-inspiratory and -expiratory lung volumes increase during exercise to facilitate an increase in V_T and expiratory flow rates (Johnson et al. 1992). Isovolumic conditions to measure changes in P_{di} are important as diaphragm length is a critical determinant of force output and, the P_{ditw} response to nerve stimulation. An increase in lung volume lengthens the inspiratory muscles and reduces their force generation capabilities (Braun et al. 1982; Hamnegard et al. 1995; Hubmayr et al. 1989; Johnson et al. 1993). When the P_{ditw} response to BAMPS was corrected for changes in lung volume P_{ditw} decreased within the initial two thirds of heavy cycling exercise, and remained reduced through to task failure (Walker et al. 2011). In summary, the diaphragm can fatigue during exercise and certain factors may contribute to exercise-induced respiratory muscle fatigue.

1.5.4 FACTORS CONTRIBUTING TO EXERCISE-INDUCED RESPIRATORY MUSCLE FATIGUE

The amount of muscle mass involved with the exercise may be a factor that contributes to exercise-induced respiratory muscle fatigue. Diaphragm fatigue has not been observed in wheelchair athletes with cervical spinal cord injury after high-intensity (>90% $\dot{V}O_{2max}$) upper-body exercise; most likely due to the relatively low amount of body mass involved in producing the ventilatory response to exercise (Taylor et al. 2010). Furthermore, respiratory muscle work alone does not seem to be the main factor contributing to exercise-induced diaphragmatic fatigue. When Babcock et al. (1995) asked participants to mimic at rest the breathing pattern (V_T

and f_B) and respiratory muscle recruitment pattern (peak transdiaphragmatic pressure; P_{dipeak}) commensurate with that attained in the final third of a prior heavy exercise test (~93% $\dot{V}O_{2max}$ for 13.2 ± 2.0 min) the P_{ditw} response to electrical bilateral phrenic nerve stimulation decreased by only 8.2 ± 3.9%. It was only when diaphragmatic force output was increased by ~1.5 to 2 times that the decrease in P_{ditw} matched that achieved during exercise (~26%). This finding implies that the diaphragms fatigue threshold increases when respiratory muscle work is undertaken at rest, and the authors' interpreted that this was due to blood flow requirements. At rest, there may be an adequate supply of blood flow available to the diaphragm whereas during heavy exercise there may be competition between the working diaphragm and locomotor muscles (Harms et al. 1997; Harms et al. 1998). This interpretation has been supported by a similar experimental design which reported that the increase in intercostal muscle blood flow that accompanies an increased ventilation and WOB was reduced during cycling, but not volitional hyperpnoea at rest (Vogiatzis et al. 2009). This competition for blood flow may reduce O_2 transport to the working respiratory muscles. Evidence from Vogiatzis et al. (2008) supports this: Trained cyclists ($\dot{V}O_{2max}$: 63 ± 4 $mL \cdot kg^{-1}min^{-1}$) exercised for 5 min during hypoxia, normoxia and hyperoxia at a $\dot{W}_{max}$ (235, 290 and 325 W) designed to elicit the same V_T, f_B and WOB during each trial. The P_{ditw} response to bilateral electrical phrenic nerve stimulation was decreased to the greatest extent in hypoxia. Moreover, limb and seventh intercostal blood flow (measured with light-absorbing tracer dye indocyanine green in combination with near-infrared spectroscopy) and cardiac output did not change during any condition. This finding suggests that during heavy exercise there is competition between the respiratory muscles and locomotor muscles for blood flow. With hypoxia there is no increase in intercostal muscle blood flow which

compromises O_2 supply, and exacerbates diaphragmatic fatigue. However, it should be noted that with the limitations of the near-infrared spectroscopy technique it is still not known whether the diaphragm receives an increase in blood flow. It is likely that this increase in diaphragmatic fatigue would compromise exercise tolerance.

1.5.5 EXERCISE-INDUCED RESPIRATORY MUSCLE FATIGUE AND EXERCISE TOLERANCE

The question of whether exercise-induced respiratory muscle fatigue may negatively impact upon exercise tolerance has been examined by fatiguing the respiratory muscles with IRL or volitional hyperpnoea before commencement of exercise. IRL can be used to preferentially fatigue the inspiratory muscles whereas volitional hyperpnoea has been used to globally fatigue the respiratory muscles. These studies have shown that exercise capacity has decreased (Mador and Acevedo 1991; Martin et al. 1982) or not changed (Dodd et al. 1989; Sliwinski et al. 1996; Spengler et al. 2000). However, these studies have either not assessed diaphragm fatigue (Dodd et al. 1989; Martin et al. 1982; Spengler et al. 2000) or not used objective nerve stimulation techniques (Mador and Acevedo 1991). Thus, it is possible that some of these studies may not have elicited inspiratory muscle fatigue before commencement of subsequent exercise, or induced fatigue that is not commensurate with exercise-induced inspiratory muscle fatigue (Romer and Polkey 2008). Moreover, a significant change in breathing pattern during the subsequent exercise has been observed, specifically an increased f_B with no change in V_T (rapid, but not shallow breathing), and a heightened sensation of both leg and breathing discomfort (Mador and Acevedo 1991; Martin et al. 1982). The elevation in f_B is caused by an increase in central motor output (Sheel et al. 2001; St Croix et al. 2000). The conscious awareness of central motor output (via corollary discharge

from the motor cortex to the sensory cortex) increases perceptions of dyspnoea (Gandevia et al. 1981) which are both important determinants of exercise tolerance (Jones and Killian 2000; Presland et al. 2005). Therefore, it is unclear whether the reduction in exercise tolerance is due to an alteration in breathing pattern, a heightened sense of dyspnoea and/or, respiratory muscle fatigue.

To avoid these confounding effects researchers have unloaded the inspiratory muscles with a PAV during exercise. Using this approach, Babcock et al. (2002) have shown that diaphragmatic fatigue was abolished after cycling exercise at ~85% $\dot{V}O_{2max}$ until volitional tolerance with a PAV where during the second half of exercise the WOB was reduced by ~50% and $\dot{V}O_2$ by ~12−13%. In contrast, when the exercise was performed without PAV for the same duration (9.6 ± 0.6 min) the WOB and $\dot{V}O_2$ were unchanged, and P_{ditw} was reduced by 20.3 ± 3.1% at the end of exercise and was still reduced 60 min into recovery.

Harms et al. (2000) reported an improvement in exercise tolerance when a PAV was used with male trained cyclists ($\dot{V}O_{2max}$: 61 ± 4 $mL \cdot kg^{-1}min^{-1}$). Participants cycled at ~90% $\dot{V}O_{2max}$ until volitional tolerance and compared to control conditions cycling capacity increased by 14% with a PAV, and decreased by 15% with IRL (Figure 1.11). However, earlier studies showed no benefit on exercise tolerance using a PAV (Gallagher and Younes 1989; Krishnan et al. 1996; Marciniuk et al. 1994). There may be several explanations for the differences in findings. Firstly, the earlier studies were undertaken at relatively lower exercise intensities (70−80% $\dot{V}O_{2max}$), which may not elicit diaphragmatic fatigue (see MECHANISMS BY WHICH RESPIRATORY MUSCLE FATIGUE MAY REDUCE EXERCISE TOLERANCE). Secondly, participants in those earlier studies had lower fitness levels compared with the participants used by Harms et al. (2000). As stated earlier, the O_2 cost of breathing

during heavy exercise is ~5% higher in trained, compared to moderately fit, participants (Aaron et al. 1992a; Aaron et al. 1992b) suggesting that reducing the WOB would be most noticeable in the highly fit participants. Finally, Harms et al. (2000) utilised several familiarisation trials and multiple randomised exercise capacity tests in an attempt to reduce the effect of random variability on exercise tolerance. It must be noted, however, that a placebo group was not used by Harms et al. (2000) which weakens internal validity and risks participant bias. These findings do still suggest that diaphragm fatigue and the WOB may influence exercise capacity. There may be several mechanisms by which respiratory muscle fatigue limits exercise tolerance.

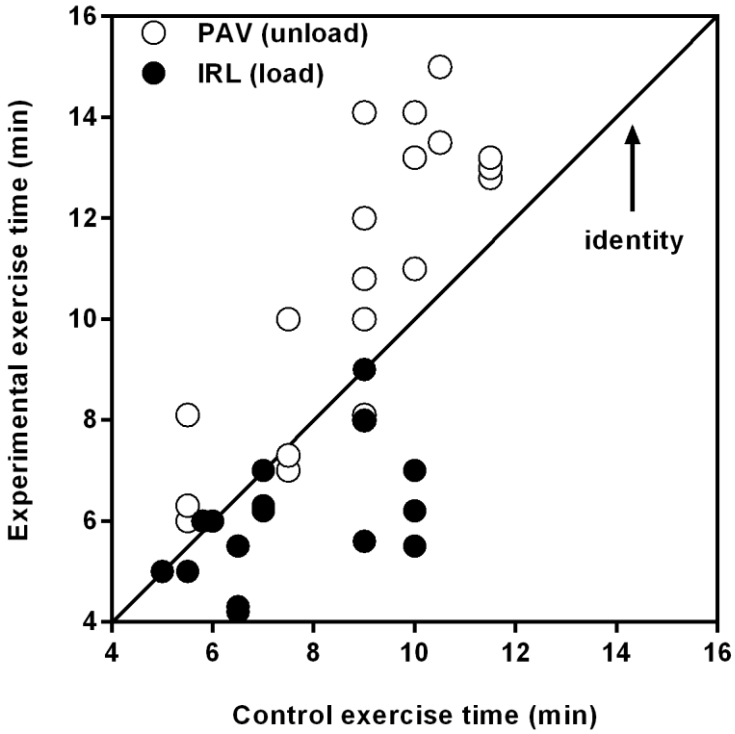


Figure 1.11 Relationship between exercise capacity time to volitional tolerance with experimentally changing the work of breathing using a proportional assist ventilator (PAV) to unload, or inspiratory flow-resistive loading (IRL) to load, the respiratory muscles. Note that with PAV 16 of 21 trials were longer than control trials, whereas with IRL 15 of 18 trials were shorter than control trials. Adapted from Harms et al. (2000).

1.5.5.1 MECHANISMS BY WHICH RESPIRATORY MUSCLE FATIGUE MAY LIMIT EXERCISE TOLERANCE

Exercise-induced respiratory muscle fatigue may limit exercise tolerance by decreasing the relative contribution of the diaphragm to total ventilation. As stated earlier (see RESPIRATORY MECHANICS AND KINEMATICS DURING EXERCISE), when exercise progresses there is a gradual increase in the recruitment of rib cage muscles to deliver the ventilatory response (Aliverti et al. 1997). This has also been demonstrated at higher intensity (80−85% $\dot{V}O_{2max}$) constant-load exercise until the limit of volitional tolerance (Babcock et al. 1995; Babcock et al. 1995; Babcock et al. 1996; Babcock et al. 1998; Babcock et al. 2002; Johnson et al. 1993). The recruitment of less efficient respiratory muscles as exercise progresses may distort the chest wall (Goldman et al. 1976; Grimby et al. 1976), impair breathing mechanics (Dodd et al. 1988; Hart et al. 2002) and thus increase the metabolic and blood flow demands of these muscles. Accessory respiratory muscle recruitment may also increase the perception of dyspnoea by increasing sensory input to the central nervous system (Romer and Polkey 2008). Respiratory muscle fatigue may also increase the severity of dyspnoea (Gandevia et al. 1981; Supinski et al. 1987; Suzuki et al. 1992; Ward et al. 1988). However, this effect seems to be localised to the accessory inspiratory muscles as diaphragm fatigue does not increase neural drive (Luo et al. 2001), and specific resistive loading of the diaphragm does not increase the perception of inspiratory effort (Bradley et al. 1986; Ward et al. 1988)

A more probable mechanism by which respiratory muscle fatigue may limit exercise tolerance are cardio-respiratory interactions during exercise. In 2002, Jerome Dempsey's research group in Wisconsin, USA, proposed that fatiguing respiratory muscle contractions cause a fatigue-induced metaboreflex (Figure 1.12) (Dempsey et al. 2002). There is compelling evidence for the metaboreflex from

animal studies. After the diaphragm or abdominal muscles are stimulated electrically, pharmacologically or infused with a bolus of lactic acid there is an increase in efferent sympathetic activity and mean arterial pressure whereas limb vascular conductance decreases (Hussain et al. 1991; Offner et al. 1992; Rodman et al. 2003). For example, when lactic acid is infused into the phrenic and deep circumflex arteries (which supply the diaphragm and abdominal muscles) of dogs at rest and during sub-maximal exercise mean arterial pressure increased by 21% and cardiac output was reduced by 6% (Rodman et al. 2003). Furthermore, limb perfusion was reduced by 20% which was caused by an increased locomotor muscle sympathetic nerve activity. These vascular responses appear to be sympathetically mediated because the effects were not observed with pharmacological blockade of the adrenergic receptors.

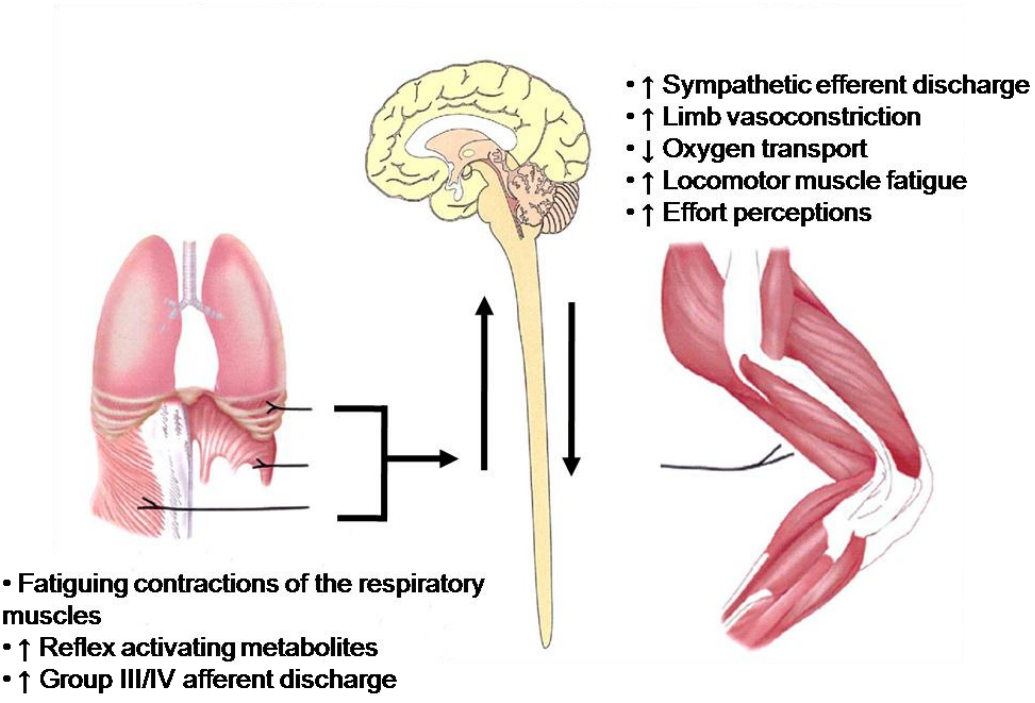


Figure 1.12 Respiratory muscle metaboreflex is started by respiratory muscle fatigue mediated supraspinally through group III/VI afferents and resulting in sympathetically mediated vasoconstriction of limb locomotor vasculature increasing peripheral locomotor muscle fatigue and (via feedback) increases the perception of dyspnoea and thus contributing to limit high-intensity exercise tolerance (Dempsey et al. 2002). Adapted from Romer and Polkey (2008).

There is evidence for the metaboreflex in humans. When participants undertake inspiratory and expiratory resistive loading at rest until task failure (or diaphragm fatigue) there is a time dependent increase in muscle sympathetic nerve activity in the resting leg; even with a compensatory increase in systemic blood pressure (St Croix et al. 2000). This is accompanied by a decrease in limb vascular conductance (femoral blood flow measured with Doppler ultrasound / mean arterial pressure) and a reduction in limb blood flow (Sheel et al. 2001; Sheel et al. 2002). In exercise the interpretation of the metaboreflex is complex as the increase in muscle blood flow depends upon the combination of vasodilators and vasoconstrictors (Romer and Polkey 2008). Harms et al. (1997) have shown an increase in vascular conductance and blood flow to the limbs when the WOB is reduced with a PAV during heavy exercise. Conversely, increasing the WOB with IRL causes the opposite effect, and there are decreases in limb vascular conductance and limb blood flow. It could be speculated that the change in limb vascular conductance was sympathetically mediated as these changes with the WOB were correlated with changes in noradrenaline spillover. However, direct recordings of sympathetic nerve activity are more accurate than noradrenaline (Folkow et al. 1983), and recently this has been undertaken during exercise (Katayama et al. 2012). Participants cycled at sub-maximal intensities (~40% peak oxygen uptake; $\dot{V}O_{2peak}$) for 15 min with spontaneous breathing throughout or IRL (5 min at MIP = 30% and $T_I/T_{TOT} = 0.5$) in the middle third of exercise. Muscle sympathetic nerve activity (via microneurography of the right median nerve at the elbow) was increased to a greater extent with IRL than spontaneous breathing (83.4 vs. 19.2%) even with a compensatory increase in systemic blood pressure (Figure 1.13). In a prior exercise trial of the same intensity, and with IRL, global inspiratory muscle fatigue (via MIP)

decreased by ~10%. These results suggest that inspiratory muscle fatigue increases muscle sympathetic vasomotor outflow (metaboreflex), and these findings have been recently confirmed (Katayama et al. 2013). However, the assessment of inspiratory muscle fatigue was measured with MIP and exercise can reduce central drive globally contaminating the interpretation of MIP (ATS/ERS 2002). Furthermore, with this small amount of global inspiratory muscle fatigue it could be speculated that, during exercise (but not at rest; Sheel et al. (2001) and St Croix et al. (2000)), muscle sympathetic nerve activity increases without fatiguing respiratory contractions. What is still unknown regarding the metaboreflex is whether it occurs during dynamic heavy whole-body exercise.

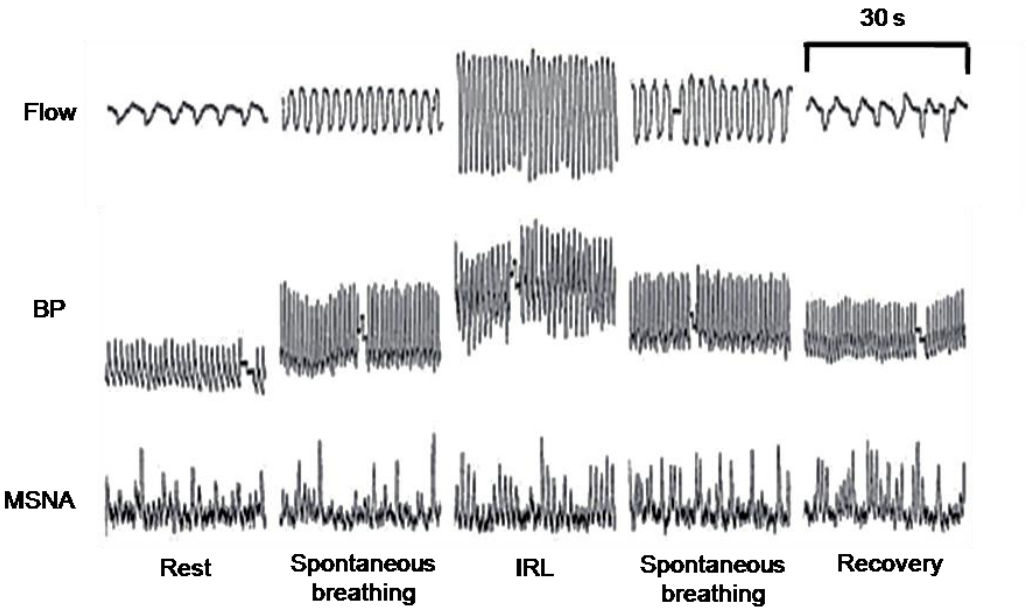


Figure 1.13 Recordings of flow, blood pressure (BP) and muscle sympathetic nerve activity (MSNA) during 15 min cycling exercise at ~40% peak oxygen uptake with 5 min of inspiratory flow-resistive loading (IRL) during the middle third of exercise. Note the increase in BP and MSNA during IRL. Adapted from Katayama et al. (2012).

The metaboreflex may also increase peripheral locomotor fatigue and the perceptions of leg effort during high-intensity (>90% $\dot{V}O_{2peak}$) exercise (Romer et al. 2006). After the respiratory muscles were unloaded with a PAV there was an

increase in cycling exercise capacity, a reduction in the amount of quadriceps fatigue (via twitch force from magnetic stimulation of femoral nerve), and a reduction in leg effort perceptions compared to control conditions of the same exercise duration (13.2 ± 0.9 min). Conversely, when respiratory muscle force output was increased with IRL there was the opposite effect, and quadriceps fatigue was exacerbated, there was a decrease in exercise duration (7.0 ± 0.6 min), and heightened leg effort perceptions. Recently, it has been shown that prior fatigue of the quadriceps can also mediate the opposite effect and increase respiratory effort sensations (Grippo et al. 2010). Whilst the metaboreflex is important during exercise; it does not play a role during respiratory muscle fatigue following resistive loading or volitional hyperpnoea (breathing-induced respiratory muscle fatigue).

1.5.6 BREATHING-INDUCED RESPIRATORY MUSCLE FATIGUE

Using nerve stimulation techniques, peripheral low frequency diaphragm fatigue has been observed after restive loading (Laghi et al. 1995; Laghi et al. 1996; Mador et al. 1996; Moxham et al. 1981; Sheel et al. 2001; Sheel et al. 2002; Travaline et al. 1997a; Travaline et al. 1997b) and volitional hyperpnoea (Babcock et al. 1995; Hamnegard et al. 1996; Mador et al. 1996; Mador et al. 2002; Rafferty et al. 1999; Renggli et al. 2008; Verges et al. 2009; Verges et al. 2010) undertaken at rest.

The limitations of IRL has been discussed previously (see WORK OF BREATHING DURING EXERCISE), and most studies have used this protocol to induce (and examine) the mechanisms of diaphragmatic fatigue. The difficulty with interpreting the findings from volitional hyperpnoea is that most, but not all (Babcock et al. 1995), have not mimicked the WOB and/or respiratory muscle recruitment patterns that are commensurate with whole-body exercise (see WORK OF

BREATHING DURING EXERCISE). When the WOB and/or respiratory muscle recruitment patterns are mimicked the degree of fatigue is reduced. For example, when Babcock et al. (1995) asked participants to mimic at rest the breathing pattern (V_T = 2.7 L and f_B = 43 breaths·min^{-1}) and respiratory muscle recruitment pattern (P_{dipeak}) commensurate with heavy exercise for ~13 min the P_{ditw} response to bilateral electrical phrenic nerve stimulation decreased by ~8%. In contrast, Renggli et al. (2008) reported that the P_{ditw} response to cervical magnetic stimulation decreased by ~20% following 8 min of volitional hyperpnoea where only V_T (~2.9 L) and f_B (~46 breaths·min^{-1}) were controlled. The differences could be explained by stimulation techniques, but most likely (as breathing pattern was similar) by an increase in end-expiratory lung volume and thus increased WOB by the participants in the Renggli et al. (2008) study. This has previously been observed when participants attempt to mimic the pressure-volume characteristics of exercise hyperpnoea (Klas and Dempsey 1989).

1.5.6.1 MECHANISMS FOR BREATHING-INDUCED DIAPHRAGM FATIGUE

The aetiology for breathing-induced diaphragm fatigue is considerably different from that observed after whole-body exercise. Metabolite accumulation (hydrogen ions, inorganic phosphates and potassium) within the respiratory muscles may explain part of diaphragmatic fatigue after high levels of work. Indeed, volitional hyperpnoea increases blood lactate concentration both at rest (Brown et al. 2008; Renggli et al. 2008; Verges et al. 2009; Verges et al. 2010), and when superimposed upon exercise (Brown et al. 2012; Johnson et al. 2006) suggesting that the respiratory muscles are capable of net lactate release. It has also been reported

that oxidative stress may play a role in breathing-induced diaphragmatic fatigue (see BREATHING-INDUCED OXIDATIVE STRESS AND RESPIRATORY MUSCLE FATIGUE).

During longer duration respiratory muscle work (with isocapnia) peripheral diaphragm fatigue may relate to a reduction in respiratory muscle glycogen content. Due to experimental limitations there is no data on the effect of increased respiratory muscle work on diaphragm glycogen content. There are, however, many well controlled animal studies showing a reduction in diaphragm glycogen content in rats after prolonged and strenuous exercise (Fregosi and Dempsey 1986; Green et al. 1987; Green et al. 1988; Ianuzzo et al. 1987; Namiot et al. 1985; Namiot and Gorski 1988). Green et al. (1987) reported a decrease (73–80%) in glycogen (measured microphotometrically using the periodic acid-Schiff technique) in the diaphragms of rats after 30 min treadmill running (25 metre·min^{-1} and 8% grade) compared to control animals. In another group of rats that continued exercising until exhaustion (which represented an additional 94 min) no further reduction in diaphragm glycogen was evident, suggesting that at least in the early phase of exercise, the respiratory muscles are dependent upon glycogen as a substrate. Supporting this, there is also a decrease in diaphragm glycogen content (~30% measured microphotometrically using the periodic acid-Schiff technique) in sheep exposed to high resistive loads (>150 $cmH_2O \cdot L^{-1} \cdot s$) (Bazzy et al. 1988). Interestingly, respiratory muscle fatigue (assessed after task failure) occurred at a time when considerable glycogen was still present in type I fibres in the diaphragm suggesting that only a small reduction is required to initiate task failure. Although not demonstrated in humans it could be speculated that a decrease in respiratory muscle glycogen, in part, contributes to breathing-induced diaphragm fatigue. The previous sections have shown that the WOB is considerable during exercise and can result in diaphragm fatigue. This can

contribute to locomotor fatigue and subsequently reduce exercise tolerance. Many experiments have used a PAV to reduce the WOB. However, the use of a PAV during exercise competition or with patients that exhibit an increased WOB at rest is impractical. As a consequence, specific training of the respiratory muscles may provide a unique alternative to potentially improve exercise tolerance.

1.6 RESPIRATORY MUSCLE TRAINING

1.6.1 HISTORICAL PERSPECTIVES

Respiratory muscle training (RMT) was first developed in clinical populations by Delhez et al. (1966) where 3 s Müller manoeuvres were repeated for 8 weeks resulting in an increased MIP of 37%. It was concluded that the increase in inspiratory muscle strength (which was a surprising finding at the time) may reduce the potential for the respiratory muscles to limit ventilatory work. The now classic study by Leith and Bradley in 1976 was the first to investigate the effects of RMT on the strength and endurance of the respiratory muscles. They observed that 5 weeks of RMT using repeated static inspiratory manoeuvres over the vital capacity range (strength training) improved MIP by 55% whereas volitional hyperpnoea training at rest (endurance training) improved maximal sustainable ventilatory capacity by 18%. This was the first study to demonstrate that known training principles apply to the respiratory muscles. These training principles still apply to modern day RMT which can be performed using voluntary isocapnic hyperpnoea (VIH), flow-resistive loading and pressure-threshold loading.

1.6.2 RESPIRATORY MUSCLE TRAINING TECHNIQUES

1.6.2.1 VOLUNTARY ISOCAPNIC HYPERPNOEA

VIH requires the participant to increase $\dot{V}_E$ to a prescribed level for a given period of time. Typical training intensities are 50−80% of the individual maximum voluntary ventilation (MVV) with f_B fixed at 30−45 breaths·min^{-1} and a V_T of 50−60% of vital capacity (~2.5 to 3.0 L). VIH is performed for ~30 min daily and for up to 4 weeks (Boutellier and Piwko 1992; Boutellier et al. 1992; McMahon et al. 2002; Morgan et al. 1987; Spengler et al. 1999). The accurate monitoring of breathing pattern is fundamental to VIH in order to maintain the prescribed training intensity. VIH causes a rapid reduction in arterial partial pressure of carbon dioxide (PCO_2). Additional CO_2 is added to the inspiratory breathing circuit to maintain isocapnia using either laboratory based equipment to monitor end-tidal and/or PCO_2 or by using a re-breathing bag. The re-breathing bag (typically fixed at 50% vital capacity) inflates during expiration and the remainder of the expirate is released into the atmosphere. In the subsequent inspiration the inspirate is comprised of both atmospheric and CO_2 enriched air from the re-breathing bag. Early studies had participants undertake VIH within the laboratory. More recently, commercially available portable devices have been made available (Figure 1.14).

VIH is used to improve respiratory muscle endurance rather than strength. It targets the velocity axis of the respiratory muscle force-velocity curve and both the inspiratory and expiratory muscles are trained. Considerable improvements in respiratory muscle endurance have been observed after VIH with Boutellier and Piwko (1992) an increase from 4.2 to 15.3 min after 4 weeks of VIH in untrained individuals. Larger increases have been reported from trained individuals after VIH with increases from 6.1 to 40 min in healthy trained individuals (Boutellier et al.

1992), 9.8 to 36.7 min in moderately trained males reporting (Verges et al. (2007) and 14.7 to 36.0 min in well-trained competitive runners (Leddy et al. 2007).

VIH has very high external validity as it mimics the ventilatory response to exercise. However, training sessions are time consuming and often require supervision and/or specialised and expensive equipment. The high flow rates required with VIH may also cause chronic dying of the airways leading to bronchoconstriction (McConnell and Romer 2004).

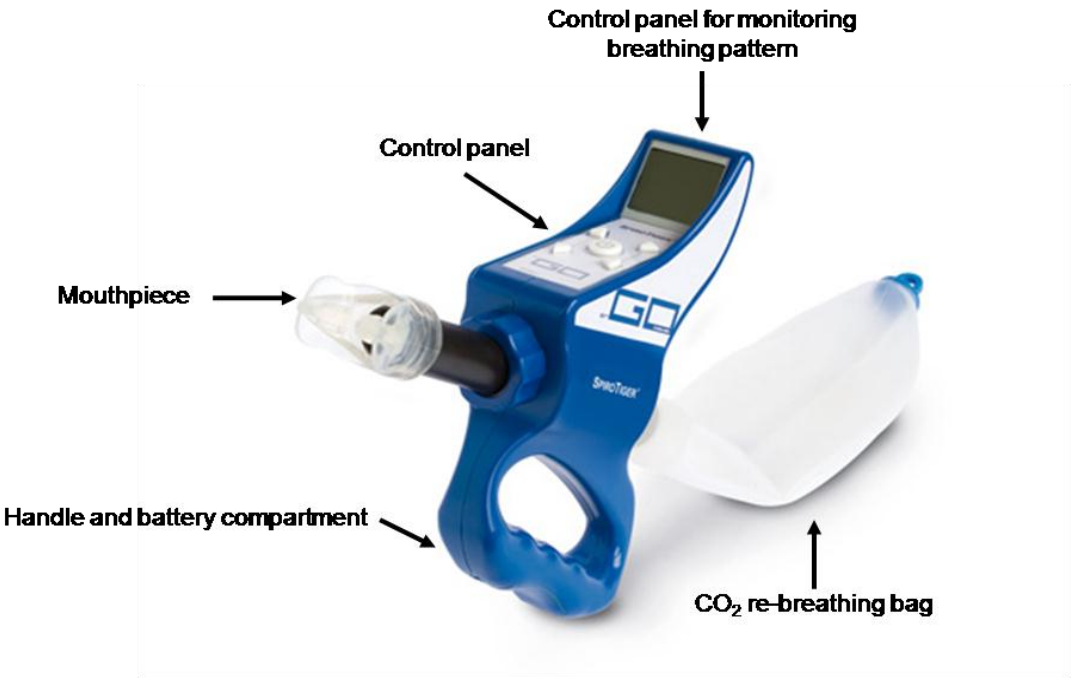


Figure 1.14 Example of commercially available portable voluntary isocapnic hyperpnoea respiratory muscle training device. CO_2, carbon dioxide. Adapted from www.spirotiger.com.

1.6.2.2 FLOW-RESISTIVE LOADING

During flow-resistive loading participants inspire through a variable sized aperture which provides resistance. During inspiration the pressure generated by the inspiratory muscles (i.e., training resistance) is dependent upon inspiratory flow rate. Thus, monitoring the breathing pattern during training is important as a decrease in

inspiratory flow rate reduces the amount of resistance and therefore inspiratory load. A novel approach to flow-resistive loading has been to use the 'TIRE' system (Test of Incremental Respiratory Endurance) where an inspiratory pressure (based on a percentage of MIP) is attained for six repetitions with 60 s recovery between inspiratory efforts. Training sessions require participants to inspire repeatedly against a load of 80−100% MIP until the target pressure can no longer be maintained and are typically for 3 days per week for 4 to 10 weeks (Enright et al. 2006; Gething et al. 2004a; Gething et al. 2004b). To increase the training intensity the recovery time between breaths is reduced (Enright et al. 2006; Gething et al. 2004a; Gething et al. 2004b).

Flow-resistive loading specifically targets the inspiratory muscles. The specific effect of flow-resistive loading on the inspiratory muscle force-velocity curve is dependent upon inspiratory flow rate and the amount of resistance. Improvements in both strength and endurance of the inspiratory muscles have been observed using the TIRE system. Improvements in MIP of 18−41% have been reported after 4 to 6 weeks (Enright et al. 2006; Gething et al. 2004a). Improvements in inspiratory muscle endurance have also been observed. For example, sustained inspiratory pressure (which requires participants to inspire maximally from residual volume to total lung capacity over 30 s) increases from 36 to 47% (Enright et al. 2006; Gething et al. 2004a). Diaphragm thickness (via B mode ultrasonography) at functional residual capacity has been reported to increase by 12% (Enright et al. 2006). Enright et al. (2006) also reported an increase in vital capacity and total lung capacity from 4.1 to 4.4 L and 5.7 to 6.1 L, respectively. It was suggested that these improvements were not due to increases in absolute lung volume but rather the

increased ability of the upper chest wall and neck inspiratory muscles to expand the thorax at greater lung volumes.

However, flow-resistive loading has weak external validity as it bears no relation to the dynamic inspiratory muscle function observed during whole-body exercise. Furthermore, the TIRE system is costly and can only be utilised within the laboratory setting by most users. The training is also physically demanding with each session lasting ~30 min.

1.6.2.3 PRESSURE-THRESHOLD LOADING

Both flow-resistive loading and pressure-threshold loading are commonly referred to as inspiratory muscle training (IMT). Pressure-threshold IMT requires the participant to produce a negative pressure to overcome a threshold load and initiate inspiration. Pressure-threshold IMT allows variable loading to determine intensity. This type of loading has been achieved by using a weighted plunger (Clanton et al. 1985a), a solenoid valve (Bardsley et al. 1993) and a constant negative pressure system (Chen et al. 1998). More recently, commercially available devices are also available with a spring-loaded poppet valve (Figure 1.15; Caine and McConnell 2000). From here on, unless otherwise stated, it will be assumed that studies have utilised this type of device. With pressure-threshold IMT, each inspiratory manoeuvre is initiated from residual volume and V_T is maximised. A rapid full inspiration minimises changes in PCO_2 and negates the use of supplemental CO_2 or a re-breathing bag. Pressure-threshold IMT is also near flow-independent (Caine and McConnell 2000). Thus, there is little requirement to monitor breathing pattern during training. Recently, another device has been made available with both an inspiratory and expiratory threshold load (IMT/EMT).

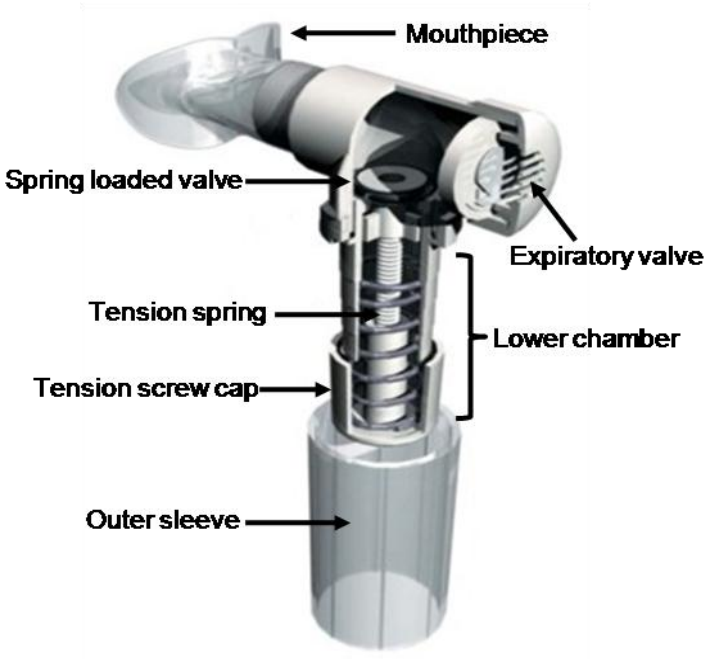


Figure 1.15 Example of commercially available pressure-threshold inspiratory muscle training device. Adapted from www.powerbreathe.co.uk.

Pressure-threshold IMT targets the inspiratory muscles. The specific effect of pressure-threshold IMT on the inspiratory muscle force-velocity (pressure-flow) curve is dependent upon the load. Romer and McConnell (2003) have demonstrated the specificity of this. Participants were assigned to different 3 groups and undertook pressure-threshold IMT (30 breaths, twice per day, 6 times per week for 9 weeks at ~50% MIP) with the following loads; high resistance-low flow (A), low resistance-high flow (B), intermediate resistance and flow (C) or no pressure-threshold IMT (D). Group A exhibited the largest increase in inspiratory pressure, group B a large increase in inspiratory flow rate, group C uniform increases in inspiratory pressure and flow rate and group D no changes in inspiratory pressure or flow rate (Figure 1.16). The most common training intensity used is intermediate resistance and flow (Brown et al. 2008; Brown et al. 2010; Brown et al. 2012; Johnson et al. 2007; McConnell and Sharpe 2005; Romer et al. 2002a). Training durations greater than 6 weeks, do not provide any further benefit (Figure 1.16). Throughout pressure-

threshold IMT the tension spring is periodically tightened to increase the training load and to accommodate the rapid improvement in MIP. This gives a very precise training load.

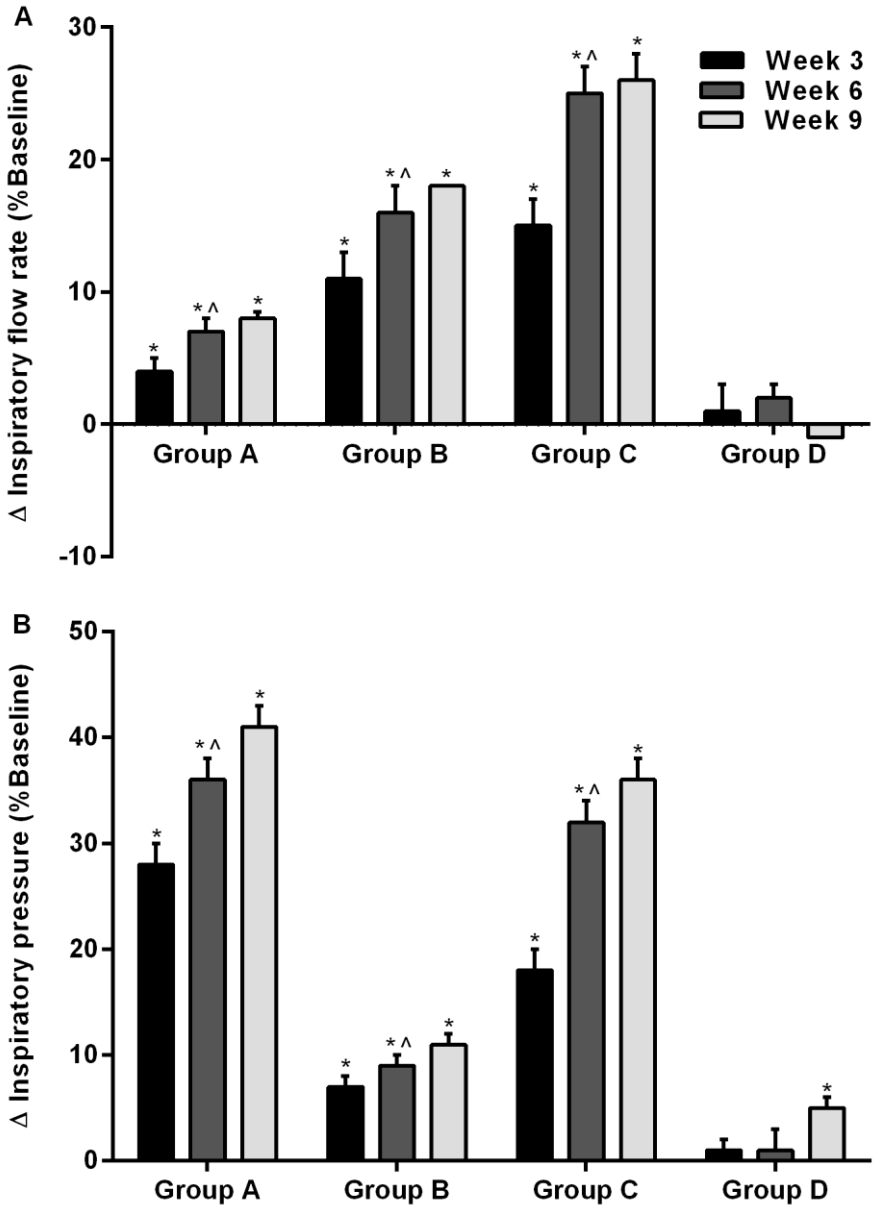


Figure 1.16 Relative changes in maximum inspiratory flow rate (A) and maximum inspiratory pressure (B) after 3, 6 and 9 weeks of pressure-threshold inspiratory muscle training (IMT) with high resistance-low flow (Group A), low resistance-high flow (Group B), intermediate resistance and flow (Group C) or no pressure-threshold IMT (Group D). Significant difference (from pre-intervention * $P < 0.05$; from preceding measurement point ^ $P < 0.05$). Values are mean ± SE. Adapted from Romer and McConnell (2003).

Increases in MIP of 17−55% have been reported after 6 weeks of pressure-threshold IMT (Brown et al. 2008; Brown et al. 2010; Brown et al. 2012; Johnson et al. 2007; McConnell and Sharpe 2005; Romer et al. 2002a). Additionally, pressure-threshold-IMT improves the pressure-flow characteristics of the inspiratory muscles. IMT increases optimal inspiratory pressure (25%), optimal inspiratory flow rate (17%) and the maximum rate of pressure development (18%) (Romer et al. 2002a). The product of inspiratory pressure and flow (inspiratory muscle power) also increases by 49% (Romer et al. 2002a). Additional reports have shown an increase in diaphragm thickness (via B mode ultrasonography) of 8−12% after IMT (Downey et al. 2007).

Pressure-threshold IMT however has weak external validity as it bears no relation to the dynamic inspiratory muscle function observed during whole-body exercise. It is, however, very cost effective, easy to use, and is less time consuming than other modes of RMT with each session usually lasting ~5 min.

1.6.3 RESPIRATORY MUSCLE TRAINING AND EXERCISE PERFORMANCE

Many studies have investigated the effects of RMT on whole-body exercise performance. Interpretation of the findings is confounded by the many different tests used to measure changes in exercise performance. Furthermore, researchers have used a variety of RMT modes, durations and intensities, and many have used an inappropriate sample size and/or lack of appropriate controls. Recently, two systematic review articles with meta-analyses have been published regarding the effects of RMT on exercise performance (Hajghanbari et al. 2012; Illi et al. 2012). Illi et al. (2012) reviewed studies that have utilised RMT to assess changes in endurance exercise performance by maximal tests (constant load, time trial, repeated

sprint and maximal incremental) in rowing, running, swimming and cycling exercise modalities.

Multiple linear regressions were undertaken on 46 original studies accepted and the model used accounted for the influence of confounders such as modality, type of RMT and test duration. The findings are summarised as follows: The overall mean difference in the effect of RMT on exercise performance between intervention and sham-training or no-training control groups was 11%, while subgroup differences were 21% for constant load tests, 13% for repeated sprint tests, and 2% for time trials and maximal incremental tests. Furthermore, repeated sprint tests (+19%) and constant load tests (+16%) detect changes in performance better than maximal incremental tests with no difference between time trials and maximal incremental tests.

The meta-analysis was undertaken on 8 controlled studies that were selected on the basis that they included a sham-training or no-training control group and that they reported the relative change and standard deviation in exercise performance. This allowed the overall difference in exercise performance between RMT and the sham/no-training control group to be calculated. Mean relative differences in exercise performance were standardised based on their standard deviations as relative improvements in time trials are generally much smaller compared with those in constant load trials. Standardised mean difference (SMD) was 1.11. A subgroup analysis of the studies revealed that performance was improved to the greatest extent when assessed by a repeated sprint test (SMD: 2.96), a time trial (SMD: 1.85), and a constant load test (SMD: 0.66). No significant improvement in performance was detected by a maximal incremental test (SMD: 0.30).

Further multiple linear regressions were also undertaken on the 46 original studies. Participants with the lowest baseline whole-body fitness levels had the largest performance improvements after RMT (6% per 10 $mL \cdot kg^{-1} \cdot min^{-1}$ decrease in $\dot{V}O_{2max}$). There were no differences in performance improvements between IMT and VIH, but combined IMT/EMT had greater performance improvements (+13% compared with IMT). With increasing test duration improvements in performance are greater (+0.4% per minute test duration), and the modality of exercise does not influence the magnitude of improvements.

It must be noted, however, that this publication has several limitations: Firstly, out of the 49 studies (only 46 accepted) that were initially selected only 28 (57%) were randomised controlled trials, 6 (12%) were controlled trials and 15 (31%) were non-controlled trials. Secondly, the median quality score of research was 2 out of 7, suggesting that the quality of work was low. A final consideration is that a funnel plot revealed potential publication bias suggesting an absence of negative smaller studies.

In summary, although the systemic review with meta-analyses from Illi et al. (2012) has limitations, it suggests the following: 1) RMT improves performance; 2) improvements in performance are best detected (in order) by repeated sprint tests, constant load tests and time trials; 3) improvements cannot be detected by maximal incremental tests; 4) the greatest improvements are in participants with the lowest baseline whole-body fitness levels; 5) the two most common RMT methods (VIH and IMT) do not differ significantly in their effect, while IMT/EMT might be superior; 6) performance is greater with longer test durations; and 7) the type of exercise modality does not influence the magnitude of improvements.

1.6.4 POSSIBLE MECHANISMS FOR IMPROVEMENT IN EXERCISE PERFORMANCE

There may be many possible mechanisms to explain the finding that RMT improves performance (Table 1.1). Research indicates that improvements in performance are not due to cardiovascular adaptations, most likely because of the moderate cardiovascular demand placed on the whole-body during RMT (Markov et al. 2001; Romer et al. 2002a; Stuessi et al. 2001).

Genuine physiological adaptations have been observed after RMT. There is an increase in the proportion of type I (38%) and size of type II (21%) fibres in the external intercostals (taken via biopsy) of chronic obstructive pulmonary disease (COPD) patients after 5 weeks of pressure-threshold IMT (Ramirez-Sarmiento et al. 2002). Potentially, this increase in respiratory muscle oxidative capacity may decrease the WOB during exercise. Indeed, using the difference between whole-body $\dot{V}O_2$ during exercise and the $\dot{V}O_2$ during volitional hyperpnoea at rest Turner et al. (2012) observed that respiratory muscle $\dot{V}O_2$ decreased by 1.5% at 75% $\dot{V}O_{2max}$ and 3.4% at 100% $\dot{V}O_{2max}$ after 6 weeks of pressure-threshold IMT. These results suggest that IMT may decrease the O_2 cost of breathing. However, the WOB was not mimicked during volitional hyperpnoea (see WORK OF BREATHING DURING EXERCISE), and it could be speculated that the decrease in the O_2 cost of breathing after IMT was related to a change respiratory muscle recruitment patterns during volitional hyperpnoea. An improvement in O_2 transport has also been reported after IMT. After 4 weeks of pressure-threshold IMT the $\dot{V}O_2$ slow component was reduced during high-intensity (0.60 to 0.53 $L \cdot min^{-1}$) and maximal cycling exercise (0.28 to 0.18 $L \cdot min^{-1}$) until the limit of volitional tolerance (Bailey et al. 2010). IMT also reduced the severity of global inspiratory muscle fatigue (via MIP). An attenuation of respiratory muscle fatigue using nerve stimulation techniques has been observed.

Verges et al. (2007) reported that 4–5 weeks of VIH attenuated the reduction in P_{ditw} response to cervical magnetic stimulation following high-intensity cycling exercise (~85% $\dot{W}_{max}$), but only in participants who demonstrated a reduction in P_{ditw} greater than 10%.

The RMT adaptations in O_2 utilisation within the respiratory muscles, improvements in O_2 delivery and reduction respiratory muscle fatigue may be involved in attenuating the respiratory muscle metaboreflex. This would improve limb blood flow (and O_2 delivery) and potentially reduce locomotor fatigue; although this is not yet investigated. Witt et al. (2007) reported that the sympathetic response to fatiguing diaphragmatic work during resistive loading (MIP = 60%, f_B = 15 breaths·min^{-1} and T_I/T_{TOT} = 0.7) is attenuated after 5 weeks of pressure-threshold IMT. Before IMT, cardiac frequency (f_C) and mean arterial pressure were 83 ± 4 beats·min^{-1} and 99 ± 3 mmHg at task failure (~9 min). When resistive breathing was undertaken for the same duration and intensity after IMT, f_C and mean arterial pressure were reduced to 74 ± 42 beats·min^{-1} and 89 mmHg, respectively.

McConnell and Lomax (2006) reported that prior fatigue of the inspiratory muscles using a similar resistive breathing task reduced subsequent isolated plantar flexion performance relative to a control trial. The reduction in performance in the control trial was similar to when a pressure cuff was fastened around the leg which reduced leg blood flow from 7.75 ± 1.70 to 6.86 ± 1.24 mL·min·mmHg^{-1}. After 4 weeks of pressure-threshold IMT (and with a similar volume of inspiratory muscle work prior to plantar flexion exercise) the time to the limit of tolerance was significantly improved. The improvement in plantar flexion exercise tolerance was attributed to an IMT-mediated improvement in leg blood flow following fatiguing inspiratory muscle work. This finding has also been demonstrated in chronic heart

failure patients where calf blood flow during fatiguing inspiratory muscle work was increased as was forearm blood flow during intense handgrip exercise after 4 weeks of pressure-threshold IMT (Chiappa et al. 2008).

These indirect measures suggest a possible role for RMT in attenuating the respiratory muscle metaboreflex during intense exercise with fatiguing diaphragmatic work. However, many studies have shown that RMT improves performance during exercise below the threshold for diaphragm fatigue and the respiratory muscle metaboreflex. For example, pressure-threshold IMT improves 20 and 40 km time trial performance where the average power output is ~75% $\dot{W}_{max}$ (Romer et al. 2002a). Thus, other possible mechanisms are likely to underpin the improvements in performance observed at sub-maximal exercise intensities. These mechanisms may be decreased systemic blood lactate concentrations ($[La^-]_B$) and/or a reduction in the perception of locomotor or respiratory effort perceptions.

Table 1.1 Possible mechanisms for the improvement in performance after respiratory muscle training.

Reference	Possible mechanism
Bailey et al. (2010)	Improved oxygen uptake kinetics
Griffiths and McConnell (2007); Romer et al. (2002c)	Decreased respiratory and limb effort perceptions
McConnell and Lomax (2006); Witt et al. (2007)	Attenuated respiratory muscle metaboreflex
McConnell and Sharpe (2005); Spengler et al. (1999)	Decreased systemic blood lactate concentration
Ramirez-Sarmiento et al. (2002)	Increase in proportion of type I and size of type II fibres in the external intercostals (COPD patients)
Turner et al. (2012)	Decreased oxygen cost of breathing
Verges et al. (2007)	Attenuated respiratory muscle fatigue

COPD, chronic obstructive pulmonary disease.

A common observation after RMT is a reduction in systemic $[La^-]_B$ which has been reported during maximal incremental cycling (Spengler et al. 1999), 6 min maximal rowing (Griffiths and McConnell 2007; Volianitis et al. 2001), constant power exercise until the limit of tolerance (Bailey et al. 2010; Boutellier and Piwko 1992; Leddy et al. 2007; Spengler et al. 1999), exercise at maximal lactate steady-state (MLSS; McConnell and Sharpe 2005) and during repeated sprint exercise (Romer et al. 2002b; Tong et al. 2008). The reductions (>2 $mmol \cdot L^{-1}$) are often similar to those observed after whole-body exercise training. Recently, several reports have also observed a reduction in $[La^-]_B$ during volitional hyperpnoea at rest following RMT (Brown et al. 2008; Verges et al. 2009). Reductions in $[La^-]_B$ after RMT may occur when trained respiratory muscles either increase their lactate uptake and metabolism or decrease efflux, or a combination of both (Spengler et al. 1999). During intense exercise a reduction in circulating metabolites may attenuate the discharge of chemosensitive afferent fibres located within the diaphragm which is known to trigger the respiratory muscle metaboreflex (Rodman et al. 2003).

During sub-maximal exercise the lower $[La^-]_B$ may be more important in attenuating the perceptions of limb discomfort. Romer et al. (2002b) observed a significant correlation between the change in systemic $[La^-]_B$ and total recovery time taken between repeated sprints, as well as the reduction in the rating of perceived exertion (RPE). Thus, favourable changes in acid-base balance may be associated with the reduction in the intensity of peripheral effort sensations.

Many studies have reported a decrease in respiratory effort perceptions after RMT (Bailey et al. 2010; Romer et al. 2002a; Romer et al. 2002b; Verges et al. 2007; Volianitis et al. 2001). Along with the previous mechanisms discussed, there may be several possibilities that reduce respiratory effort perceptions during exercise. The

enhancement in respiratory drive that initiates ventilation and which is detected by the sensory brain centres intensifies the perception of dyspnoea (el-Manshawi et al. 1986). However, after pressure-threshold IMT the pressure generated during first 0.1 s of inspiration (providing a measure of respiratory muscle motor drive) decreases by 22% (Huang et al. 2003). Kellerman et al. (2000) also reported a reduction in motor output to the respiratory muscles and the magnitude estimation of the inspiratory load to varying amounts of resistive loads after 4 weeks of pressure-threshold IMT. Kellerman et al. (2000) attributed the findings to an attenuated discharge of respiratory muscle mechanoreceptors. Throughout repeated bouts of RMT sensory afferents within the respiratory muscles may become desensitised. Repeated exposures to high metabolite concentrations which may occur during RMT would reduce the afferent-mediated efferent response to a given change in metabolite concentration during subsequent exercise (Sinoway et al. 1992; Sinoway et al. 1993). Improvements in inspiratory muscle strength may also reduce the absolute force generated (Kellerman et al. 2000) and detected (Redline et al. 1991) for a given ventilation.

The improvement in performance after RMT may be related to one, or many, of the mechanisms discussed. It is most probable that they are all linked. McConnell and Romer (2004) have suggested that the improvement in performance after RMT may relate to a reduction in the discomfort associated with high levels of respiratory muscle work and/or fatigue. They hypothesise that the increase in respiratory muscle strength and/or fatigue resistance after RMT may decrease inhibitory feedback from respiratory muscles during exercise. This may delay the recruitment of less efficient accessory muscles, reduce operating lung volumes, and/or reduce the fraction of maximum tension generated with each breath. Potentially, this may reduce

respiratory muscle metabolite accumulation, and/or minimise the effects of the respiratory muscle metaboreflex, while leading to reductions in both limb fatigue and peripheral effort sensations and therefore improving performance. Whilst this is an interesting hypothesis it is yet to be proven. Since there is not one specific mechanism that can be attributed to the improvement in performance after RMT, and since performance is not always improved, it has resolved in controversy surrounding the area.

1.6.5 RESPIRATORY MUSCLE TRAINING AND CONTROVERSY

Controversy has surrounded the benefits of RMT on improving inspiratory muscle strength (Caine and Sharpe 2002; Hart et al. 2001; McConnell et al. 2002; Polkey and Moxham 2004) and exercise tolerance (McConnell and Romer 2004; McConnell 2012; Patel et al. 2012). It is commonly understood that changes in inspiratory muscle strength measured via MIP do not correlate with improvements in performance. Therefore, this debate will not be discussed. The debate regarding the ergogenic benefit of RMT on exercise tolerance has two opposing views; those who feel there is no benefit of RMT and those who feel the marginal gains are worthwhile for the small effort involved. This debate most likely stems from two issues: Firstly, there is not one single attributable mechanism to the improvement in performance unlike whole-body exercise training (e.g., increased $\dot{V}O_{2max}$); and secondly, unlike whole-body exercise training performance is not always improved, and if it is the gains are marginal. The issue may relate to study design. An improvement in performance will be only shown if the exercise outcome is sensitive to RMT and the sample size is large enough to demonstrate an effect (Illi et al. 2012). Furthermore, the study should be designed with the appropriate controls. For studies using clinical

populations that are unaccustomed to exercise this may require many participants. In contrast, in studies with reliable, trained individuals a smaller amount may be required. Alternatively, like most interventions (e.g., supplements), RMT seems to have responders and non-responders (Guenette et al. 2006) and, therefore, an effect may not be observed in all participants. For clinicians using RMT to improve a patient's tolerance to exercise this may not be acceptable as a "treatment" should either work or not and if it does the mechanisms should be evident. Until a study either demonstrates a conclusive mechanism, or an effect on performance with a large sample size, this debate may continue. One area of RMT that has received little attention is the ageing population. This is surprising since the ageing respiratory system undergoes significant changes which RMT may improve.

1.7 THE RESPIRATORY SYSTEM IN HEALTHY AGEING

Three physiological events can account for most of the changes in the respiratory system during healthy ageing: 1) a progressive increase in lung compliance; 2) a progressive decrease in chest wall compliance; and 3) a progressive decrease in the strength of the respiratory muscles.

Lung static elastic recoil pressure measured in 20 to 60 year olds was reported to progressively decease at an average rate of 0.1 to 0.2 cmH_2O per year (Turner et al. 1968). The factors that contribute to this may be changes in the spatial arrangement and/or cross linking of the elastic fibre network (Fukuchi 2009). This becomes more pronounced after 50 years of age and may lead to a homogenous enlargement of air spaces causing a reduction in alveolar surface area (Fukuchi 2009). These changes functionally resemble emphysema, but histologically they are not associated with evidence of a destruction of alveolar walls (Verbeken et al.

1992). The decrease in parenchymal elasticity increases lung compliance and the static pressure-volume curve is shifted to the left and a steeper slope (Turner et al. 1968).

Chest wall compliance was reported to decrease (~31%) with age in 50 participants from 24 to 75 years (Estenne et al. 1985). Chest wall stiffening may be related to calcifications of the costal cartilages and chrondosternal junctions, or degenerative joint disease of the dorsal spine as these are often observed in chest radiographs (Edge et al. 1964). Chest wall mechanics are also changed by alterations in the shape of the thorax. Osteoporosis can result in partial (wedge) or complete (crush) vertebral fractures leading to increased dorsal kyphosis and anteroposteriordiamater (barrel chest) (Cummings and Melton 2002; Gunby and Morley 1994).

The reduction in chest wall compliance is somewhat greater than the increase in lung compliance. Therefore, total respiratory system compliance decreases and is ~20% less in an individual of 60 years compared to an individual of 20 years of age (Turner et al. 1968). This adaptation does not change total lung capacity. However, at relaxed end-expiration the rate of the decrease in lung recoil with ageing exceeds that of the chest wall so that functional residual capacity increases by 1 to 3% per decade (Zaugg and Lucchinetti 2000). Since total lung capacity remains unchanged, there is an increase in residual volume of 5 to 10% per decade which results in air trapping and a decrease in vital capacity of 20 to 30 mL per year (Zaugg and Lucchinetti 2000). The increase in residual volume is due to the loss of elastic recoil, and to a lesser extent a change in respiratory muscle function.

1.7.1 RESPIRATORY MUSCLE FUNCTION IN HEALTHY AGEING

Respiratory muscle function is impaired by the concomitant changes in geometric modifications to the rib cage, decreased chest wall compliance and increase in functional residual capacity. The remodelling of the chest wall with ageing flattens the curvature of the diaphragm shifting its length-tension relationship to a shorter length, placing the diaphragm at a mechanical disadvantage and decreasing its force generating capabilities (Edge et al. 1964; Polkey et al. 1997; Polkey et al. 1998).

Indeed, inspiratory muscle strength has been shown to decrease with age whether measured volitionally, or objectively using non-volitional nerve stimulation techniques. A study of 668 ambulatory participants aged 20 to 90 years of age showed that MIP for males and females decreased from 117.6 and 79.5 cmH_2O below the age of 40 years to 66.0 and 45.5 cmH_2O above the age of 75 years (Harik-Khan et al. 1998). Tolep et al. (1995) also reported that P_{dipeak} during a maximum Müller manoeuvre was 25% lower in older (~68 years) compared to younger (~24 years) adults. Lastly, Polkey et al. (1997) measured the paired P_{di} response to cervical magnetic stimulation and the sniff P_{dipeak} during a Müller manoeuvre in 15 older (~73 years) and 15 younger (~29 years) adults. Both P_{di} (-23%) and sniff P_{di} (-13%) were lower in older adults.

In addition to the reduction in inspiratory muscle strength there is also a decrease in inspiratory muscle endurance and an increase in the O_2 cost of breathing The maximum sustained inspiratory pressure at task failure after incremental pressure-threshold loading (5 cmH_2O increases every 2 min with breathing pattern controlled) was significantly correlated with age in 60 participants aged 20 to 80 years, demonstrating that time to task failure was lower in older adults (Johnson et al.

1997). However, it could be speculated that changes in central drive during ageing made these participants susceptible to task failure earlier. Respiratory muscle $\dot{V}O_2$ at rest was also significantly correlated with age ($r = 0.77$, $P < 0.01$) in 37 males aged 23 to 77 years of age, indicating that the O_2 cost of breathing increases with age (Takishima et al. 1990).

Apart from the remodelling of the chest wall the factors that contribute to the age-related changes in respiratory muscle function are poorly understood. In humans there is very limited data, but it has been suggested that the decrease in inspiratory muscle strength is related to nutritional status which is often deficient in ageing. A study of 4443 ambulatory participants showed that there are significant correlations between MIP and lean body mass (measured with bioelectrical impedance) or total body mass (Enright et al. 1994). Alternatively, it could be speculated that age-related changes in respiratory muscle function are due to morphological changes. However, Caskey et al. (1989) evaluated diaphragm muscle thickness using computed tomography in 120 participants from the ages of 30 to 80 years and reported that it did not change significantly with increasing age. In contrast, Marzani et al. (2005) reported that Type I fibres (performed histochemically using myofibrillar ATPase staining) in the rectus abdominis increased from 44% in adults aged 18 to 48 to 59% in older adults aged 66 to 99 years.

The data regarding morphological changes in the diaphragms of ageing rats compared to younger controls is inconsistent. There are inconsistencies regarding fibre types (Table 1.2). Furthermore, muscle fibre cross sectional area does not change (Kavazis et al. 2012; van Lunteren et al. 1995) and diaphragm weight either increases (Powers et al. 1996; Smith et al. 2002), or does not change with ageing (Criswell et al. 1997; Criswell et al. 2003; Powers et al. 1991). Interestingly, it has

been demonstrated with *in vitro* isolated diaphragm muscle strips that there is an increase in the maximum shortening of older compared to younger rats (Powers et al. 1996). The decrease in inspiratory muscle strength could be explained, in part, by an age-related increase in the water content of the diaphragm and a decrease in myofibrillar protein concentration. Criswell et al. (1997) reported that maximal tetanic force (normalised to the cross-sectional area) of *in vitro* diaphragm strips was 16.4% lower in the diaphragms of older rats. There was, however, no difference in total diaphragm mass between younger and older rats, but an age-related increase in the water content of the diaphragm and a decrease in myofibrillar protein concentration. Recently, using three-dimensional confocal microscopy and histochemical staining it has been reported that in the diaphragms of ageing rats there is a decreased motor endplate, especially on type IIb/x fibres (Suzuki et al. 2009). These morphological changes may contribute to the decreased force production and selective denervation of type IIb/x muscle fibres with aging. The decrease in inspiratory muscle endurance with ageing is also difficult to explain as the activity of oxidative enzymes in the diaphragms of ageing rats is either higher (Powers et al. 1992a; Powers et al. 1992b) or lower (Smith et al. 2002) than younger controls. The inconsistent findings with ageing rats may be related to differences in rat breed, age at sampling, or methodological techniques. Furthermore, the findings in the respiratory muscles are inconsistent with the locomotor muscles of humans where there is a decrease in the number, and cross sectional area, of type II fibres in the vastus lateralis (Lexell 1995). This may be due to the load bearing work undertaken by the locomotor muscles.

Table 1.2 Fibre type changes performed histochemically using myofibrillar ATPase staining or myosin heavy chain isoforms in the diaphragms of aging rats compared with younger controls.

Reference	Fibre type change with ageing			
	Type I	Type II	Type IIa	Type IIb/x
Eddinger et al. (1985)	↑	N/A	↓	↔
Gosselin et al. (1992b)	↔	↔	N/A	N/A
van Lunteren et al. (1995)	↓	↑	N/A	N/A
	Myosin heavy chain isoform change with ageing			
	Type I	Type II	Type IIa	Type IIb/x
Gosselin et al. (1992a)	↑	↓	N/A	N/A
Gosselin et al. (1994)	↔	N/A	↑	↑
Powers et al. (1996)	↔	N/A	↓	↑
Smith et al. (2002)	↔	N/A	↓	↑

N/A, not applicable

1.7.2 RESPIRATORY MUSCLE TRAINING IN AGEING

To date, four published studies have examined the effects of RMT in older adults (Table 1.3). After IMT or IMT/EMT there was an increase in MIP of 21−39% (Aznar-Lain et al. 2007; Huang et al. 2011; Watsford and Murphy 2008). Belman and Gaesser (1988) and Watsford and Murphy (2008) both observed changes in pulmonary function after RMT. Belman and Gaesser (1988) an increase in vital capacity after VIH, and Watsford and Murphy (2008) forced expiratory volume in 1 s (FEV_1) and peak expiratory flow (PEF) after IMT/EMT. In younger adults the changes following VIH or IMT/EMT in these pulmonary function measures are equivocal (Griffiths and McConnell 2007; Leddy et al. 2007; Verges et al. 2007; Wells et al. 2005). In contrast, FVC is rarely, and PEF never, changed after IMT in younger adults (Brown et al. 2008; Brown et al. 2010; Brown et al. 2012; Johnson et al. 2007). Improvements in respiratory muscle endurance have also been observed. Belman and Gaesser (1988) observed an increase in maximum sustainable

ventilatory capacity and MVV. Watsford and Murphy (2008) also observed an increase in MVV and incremental pressure-threshold loading. Exercise capacity was shown to be unchanged following maximal incremental exercise (Aznar-Lain et al. 2007; Belman and Gaesser 1988) which is consistent with younger adults (Illi et al. 2012). Exercise capacity did increase during constant load exercise (Aznar-Lain et al. 2007) and during a time trial (six minute walking test; 6MWT) (Huang et al. 2011). Perception of effort during exercise was equivocal with one study observing a decrease (Watsford and Murphy 2008), or no change (Belman and Gaesser 1988) after RMT. Other findings include an increase in physical activity levels (PAL) and quality of life (QoL) measures. Using accelerometers to record quantitative PAL, Aznar-Lain et al. (2007) reported an increase in moderate to vigorous physical activity (MVPA). Aznar-Lain et al. (2007) suggested this was due to an increase in the intensity of exercise performed as no other accelerometry measure changed after IMT. Huang et al. (2011) also observed an increase in the physical subcategory of the Short Form-36 (SF-36) QoL questionnaire.

Table 1.3 Respiratory muscle training studies using older adults.

Reference	Participants (Male/Female)	Age (years)	RMT	Intervention duration	RMT frequency	RMT duration	RMT Intensity	Exercise tests	Outcomes
Aznar-Lain et al. (2007)	2/7	68.5 ± 6.3	IMT	8 wk	3–5 x wk	8–10 sets / 5–6 reps	50% MIP	1. Maximal incremental treadmill 2. Exercise capacity at first ventilatory threshold	↑ MIP ↔ exercise capacity to 1 ↑exercise capacity to 2 ↑MVPA
Belman and Gaesser (1988)	7/5	69.7 ± 0.8	VIH	8 wk	4 x wk	2 x 15 $min \cdot d^{-1}$	70% MVV	1. Maximal incremental treadmill 2. 12 min at 70% $\dot{V}O_{2max}$ treadmill	↑ respiratory endurance (MSVC and MVV) ↑ VC ↑ ventilatory reserve ↔ exercise capacity to 1 ↔ in cardiorespiratory responses to 1 and 2 ↔ in RPE leg and dyspnoea
Huang et al. (2011)	22/2	70.6 ± 4.8	IMT	6 wk	5 x wk	4 sets / 6 reps	75% MIP	1. 6MWT	↑ MIP ↑ 6MWT distance ↓ RPE dyspnoea ↑ QoL
Watsford and Murphy (2008)	0/13	64.8 ± 2.5	IMT/ EMT	8 wk	12 x wk	Mixed sets / reps	30% MIP	1. 6 min incremental treadmill	↑ FVC, PEF, MIP and MEP ↑ respiratory endurance (ITL) ↓ RPE dyspnoea ↑ ventilatory reserve

6MWT, six minute walk test; EMT, expiratory muscle training; IMT, inspiratory muscle training; ITL, incremental pressure-threshold loading; MEP, maximal expiratory pressure; MIP, maximal inspiratory pressure; MSVC, maximum sustainable ventilatory capacity; MVPA, moderate to vigorous physical activity; MVV, maximum voluntary ventilation; PEF, peak expiratory flow; QoL, quality of life; RPE, rating of perceived exertion; VC, vital capacity; VIH, voluntary isocapnic hyperpnoea

Taken together, these studies suggest that RMT is effective in older adults and there are increases in inspiratory muscle strength and endurance, pulmonary function, exercise capacity (in outcome tests that are sensitive to RMT), PAL, QoL and a decrease in effort perceptions during exercise. Nevertheless, these studies have risked participant bias by failing to employ a placebo group (Belman and Gaesser 1988; Huang et al. 2011; Watsford and Murphy 2008), or are limited by a small sample size which may incur a type 2 error (Aznar-Lain et al. 2007).

A novel area in exercise and respiratory physiology has been the study of the inflammatory response to exercise. Recent data also indicates that during increased levels of work the respiratory muscles also produce and release inflammatory markers. Whether RMT can change this response is unknown. The thesis will now turn its focus on to this area.

1.8 INFLAMMATION

The immune system is a network of cells, proteins, tissues, and organs that cooperate to defend the body against attacks from elements that are foreign. The primary function of the immune system is to maintain homeostasis. This is achieved through the recognition and elimination of noxious stimuli (e.g., invading pathogens), and the repair and removal of damaged or abnormal tissue. This can occur during sepsis, trauma or disease. When the cellular elements of the immune system are challenged by noxious stimuli there is a local response known as inflammation. At the site of inflammation there is the initiation the acute-phase response.

1.9 THE ACUTE-PHASE RESPONSE AND ACUTE-PHASE PROTEINS

The acute-phase response is characterised by fever, altered vascular permeability and metabolic and pathological changes, and typically lasts between 24 to 72 h (Baumann and Gaulde 1994). The cascade of the acute-phase response is initiated and coordinated by a range of cells and inflammatory mediators. At the local site of the sepsis, trauma or disease by macrophages, and systemically in the blood by monocytes (Baumann and Gaulde 1994). These cells produce cytokines which stimulate adjacent cells in the surrounding tissue to release additional cytokines and chemokines, and initiate inflammation and infiltration. Cytokines released in the systemic circulation also stimulate the production of hepatic acute-phase proteins.

Acute-phase proteins can be classed as positive or negative depending on if their plasma concentration increases (positive) or decreases (negative) by at least 25% during the acute-phase (approximately the first 7 days) of inflammatory conditions (Gabay and Kushner 1999; Morely and Kushner 1982; Sheet and Whitehead 1994). The physiological significance of a decrease of certain plasma proteins during the acute-phase response is not fully understood, but it has been suggested that negative acute-phase proteins may be non-essential for the protection of the host during inflammatory conditions (Gabay and Kushner 1999). Positive acute-phase proteins have a variety of physiological functions such as destroying or inhibiting the growth of microbes, restricting the inflammatory response, providing coagulations factors and contributing to the innate immune system by increasing vascular permeability and chemoattracting phagocytic cells (Gabay and Kushner 1999). Negative acute phase proteins may allow amino acids to focus on producing positive acute-phase proteins more efficiently (Gabay and Kushner 1999).

1.10 THE CYTOKINE

The word ‘cytokine’ originates from the Greek word ‘cyto’ meaning cell and ‘kine’ meaning movement. Cytokines are simple peptides, proteins or glycoproteins with a molecular weight ranging from 6000 to 60,000 kDa (Akira et al. 1993). They are produced by, and communicate with, immune and non-immune cells, organs and organ systems throughout the body. The cytokine family also includes chemokines which are small cytokines or proteins (8−10 kDa) (Luster 1998). The name chemokines is based on their ability to chemoattract leukocytes.

When cytokines are produced they influence target cells. The type of target cell chosen by a particular cytokine is determined by the type of specific receptor on its surface. The receptor may be present on the surface of the same cell that produces the cytokine. This is termed autocrine regulation - regulating the activity of a cell by its own product (Figure 1.17A). Alternatively, the target receptor may be present only on the surface of another cell and not on the producer cell. If the target cell resides close to the producer cell this is termed paracrine regulation - regulating the activity of a different cell by its product (Figure 1.17B). Also, a producer cell can exert its actions on target cells that reside in distant parts of the body or in other tissues or organs. This is similar to the action of polypeptide hormones and is termed endocrine regulation (Figure 1.17C).

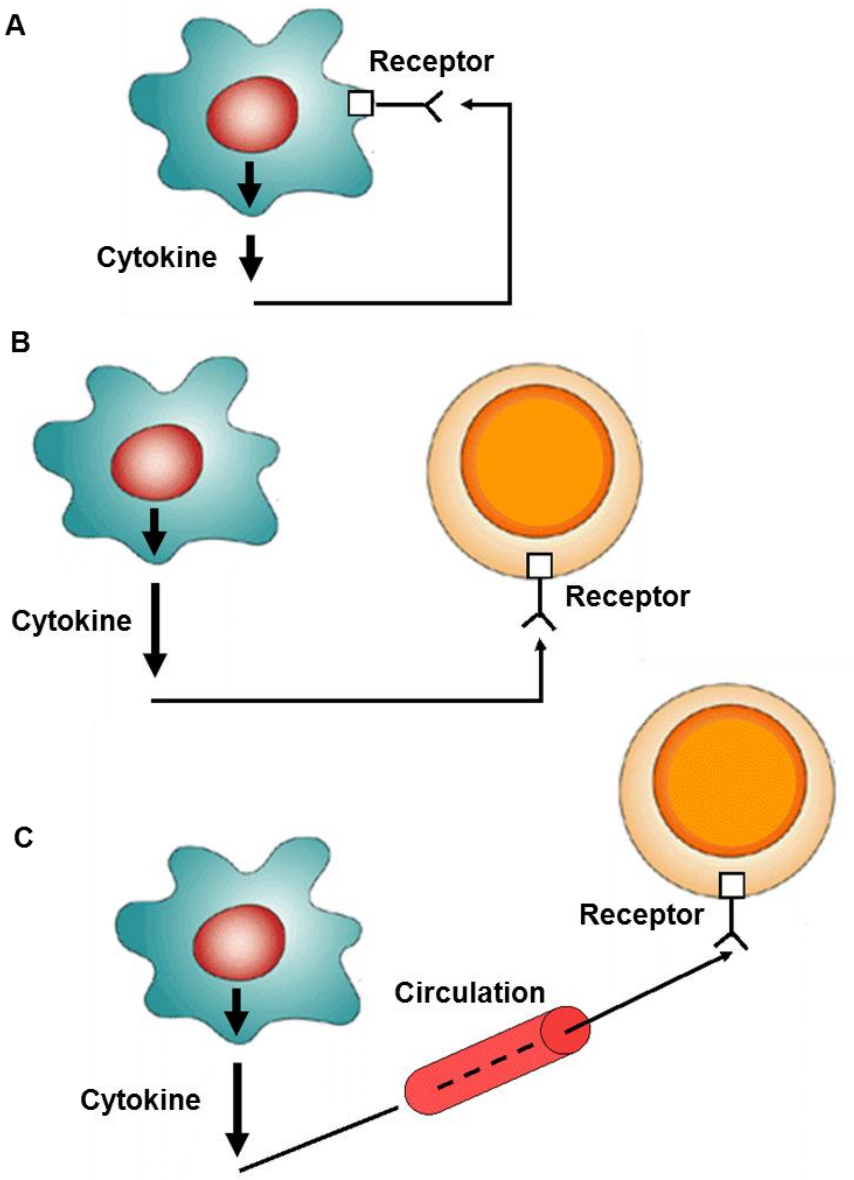


Figure 1.17 Regulation of cytokines. A) autocrine regulation; B) paracrine regulation; C) endocrine regulation.

1.10.1 MEASUREMENT OF CYTOKINES

Although the gene expression of cytokines were not measured in the experimental chapters of this thesis these techniques have been important in determining the cellular sources of cytokines that will be described in more detail later. Thus, a short overview will be given: Messenger ribonucleic acid (mRNA) can be measured with reverse transcription polymerase chain reaction (RT-PCR), Northern blotting or microarrays. The protein expression of cytokines can be

measured with several techniques including Western blot, histochemical staining or by enzyme linked immunosorbant assay (ELISA).

The ELISA has become the ‘gold standard’ in measuring systemic (blood-borne) cytokine concentrations. ELISA is a biochemical technique used to detect the presence of an antigen (cytokine) in a sample (Figure 1.18). ELISA is simple and reliable, and the development of commercially available kits has been the stimulus for a plethora of research into the cytokine response to exercise. From here on unless otherwise stated, it will be assumed that systemic cytokine concentrations have been measured using ELISA.

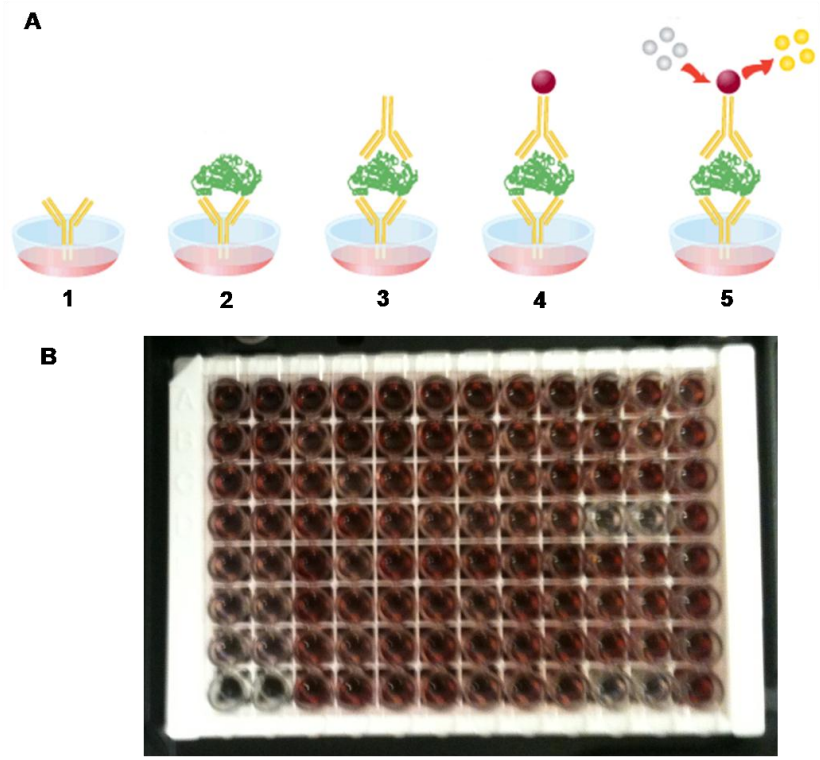


Figure 1.18 A) Sandwich enzyme linked immunosorbant assay (ELISA). 1) A surface is prepared to which a known quantity of capture antibody (specific for the cytokine) is bound; 2) the antigen-containing sample (e.g., plasma) is added to the plate; 3) a specific antibody is added, and binds to the antigen (sandwich); 4) enzyme-linked secondary antibodies are applied; and 5) a chemical is added to be converted by the enzyme into a colour or fluorescent signal and the absorbency signal of the plate wells is measured to determine the presence and quantity of the cytokine (B). Note the left hand side wells increase in colour concentration and thus cytokine concentration.

1.10.2 SYSTEMIC CYTOKINE RESPONSE TO EXERCISE

Using ELISA the systemic response of various plasma cytokines to prolonged and strenuous exercise has been observed (Figure 1.19). During exercise such as a marathon there is a substantial (100-fold) increase in the plasma concentration of interleukin-6 (IL-6) which is followed by cytokine inhibitors interleukin-1 receptor antagonist (IL-1ra), soluble tumour necrosis factor-α receptor (sTNF-αr) and the anti-inflammatory cytokine interleukin-10 (IL-10) (Ostrowski et al. 1999). Significant increases also occur in the concentrations of chemokines interleukin-8 (IL-8), macrophage inflammatory protein-α (MIP-α) and macrophage inflammatory protein-1β (MIP-1β) (Ostrowski et al. 2001). Only very prolonged exercise with an eccentric component results in a small increase in the plasma concentrations of classical pro-inflammatory cytokines interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) (Ostrowski et al. 1999). This is markedly different to the cytokine response to infections where there is a dramatic increase in TNF-α (Pedersen and Febbraio 2008). The primary cytokine investigated in response to exercise has been IL-6. This is due to the large increase IL-6 during and in recovery from prolonged and strenuous exercise

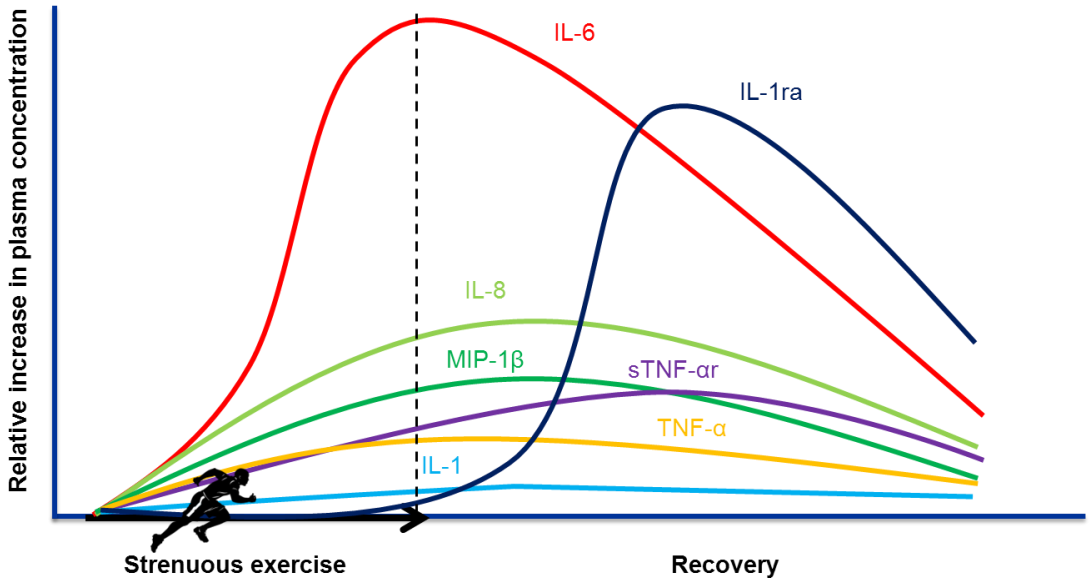


Figure 1.19 Plasma cytokine response during and in recovery from prolonged and strenuous exercise such as a marathon. IL-1, interleukin-1; IL-1ra, interleukin-1 receptor antagonist; IL-6, interleukin-6; IL-8, interleukin-8; MIP-1β, macrophage inflammatory protein-1β; sTNF-αr, soluble tumour necrosis factor-α receptor; TNF-α, tumour necrosis factor-α. Adapted from Pedersen and Toft (2000).

1.11 INTERLEUKIN-6

IL-6 is part of a family of cytokines which includes interleukin-11, oncostatin M, leukemia inhibitory factor, ciliary neurotrophic factor, cardiotrophin-1 and cardiotrophin-like cytokine (Heinrich et al. 2003). IL-6 is a glycoprotein with a molecular mass of 22–27 kDa, but this can be dependent upon the cellular source of IL-6 and the amount of post-translation modification (Pedersen et al. 2001).

During muscular contraction IL-6 signals by binding to type 1 plasma membrane receptor complexes containing the transducing receptor chain glycoprotein 130 homodimer and ligand binding chain (gp130rα/IL-rβ) (Pedersen and Febbraio 2008). It has been recently shown that c-Jun N-terminal kinase/activator protein-1 (JNK/AP-1) signalling controls IL-6 transcription in contracting skeletal muscle rather than the classical view of inflammatory IKKβ/NFκβ signalling (Whitham et al. 2012).

1.11.1 EFFECTS OF DURATION, INTENSITY AND MODALITY

Overall, the combination of duration, intensity, and modality of the exercise determines the magnitude of the exercise-induced IL-6 increase. Fischer (2006) has shown that the single most important factor in determining the plasma IL-6 response to exercise is duration (Figure 1.20). The author reviewed 74 exercise trials using 4 different modalities and ~800 participants. Linear regression of the data demonstrated that 51% of the variation in the plasma IL-6 increase can be explained by the duration of exercise. This review has also been recently supported experimentally by the finding that plasma IL-6 concentration is higher following a full- compared to a half-distance marathon (Reihmane et al. 2012).

Scott et al. (2011) have recently reported that the plasma IL-6 response to exercise is also dependent upon intensity. Plasma IL-6 concentration immediately after 1 h of treadmill running was higher at an intensity of 75% $\dot{V}O_{2max}$ compared to intensities of 65 or 55% $\dot{V}O_{2max}$. Furthermore, although not statistically significant, the plasma IL-6 concentration was higher at 65% compared to 55% $\dot{V}O_{2max}$.

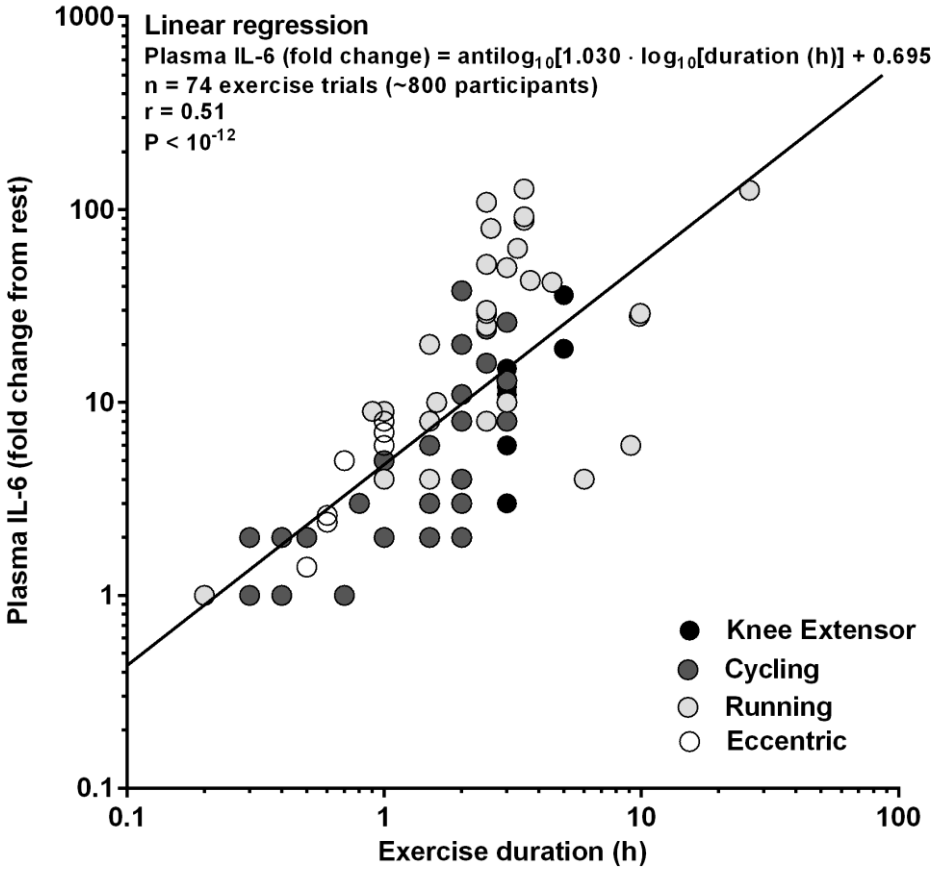


Figure 1.20 The overall $\log_{10}$-$\log_{10}$ linear relation between exercise duration and fold change in plasma interleukin-6 (IL-6). Based on data from 74 exercise trials using 4 different modalities and ~800 participants. Adapted from Fischer (2006).

It was initially hypothesised that IL-6 release was related to muscle damage, and that a greater IL-6 response may occur after an eccentric rather than a concentric modality (Bruunsgaard et al. 1997). This hypothesis was based on two observations: Firstly, when the duration and intensity of exercise was identical systemic IL-6 concentrations were higher following primarily eccentric exercise, and lower following primarily concentric exercise; and secondly, eccentric (but not concentric) exercise was significantly correlated to markers of muscle damage such as creatine kinase (Bruunsgaard et al. 1997). However, research using primarily eccentric exercise modalities (Croisier et al. 1999; Ostrowski et al. 1999; Starkie et al. 2001a), and a modified cycle ergometer to perform purely eccentric exercise (Toft et al.

2002), has now conclusively shown that eccentric exercise is not associated with a higher plasma IL-6 concentration than exercise involving non-damaging muscle contractions (Figures 1.20 and 1.21). Rather, the amount of muscle mass involved with the modality is important, as when larger muscle groups are utilised as in running or swimming, there is a greater systemic IL-6 response (Gomez-Merino et al. 2006; Nieman et al. 1998). This is primarily due to the source of IL-6 during exercise.

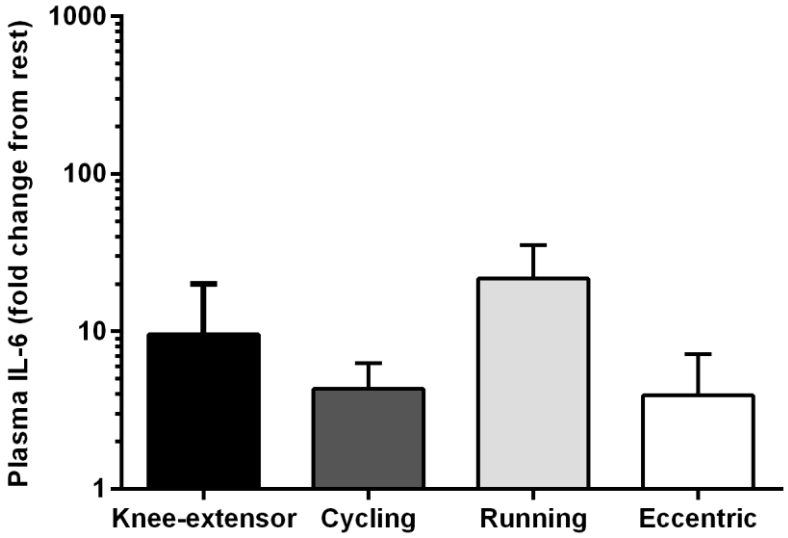


Figure 1.21 Different modes of exercise and the corresponding increase in plasma interleukin-6 (IL-6). Based on data from 74 exercise trials using 4 different modalities and ~800 participants. Values are mean $\log_{10}$-$\log_{10}$ ± SD. Adapted from Fischer (2006).

1.11.2 SOURCE OF INTERLEUKIN-6 DURING MUSCULAR CONTRACTIONS

A variety of cells can be activated to express IL-6 *in vivo*. The primary sources include activated monocytes and macrophages, fibroblasts and endothelial cells; with other cellular sources being T and B cells, neutrophils, eosinophils, osteoblasts and keratinocytes (Akira et al. 1993). Monocytes and macrophages are classed as peripheral blood mononuclear cells (PBMC) and are the major sources of IL-6 during sepsis, trauma or disease (Akira et al. 1993). However, it has been

consistently demonstrated that PBMC (Ullum et al. 1994), and in particular monocytes (Starkie et al. 2000; Starkie et al. 2001b), do not produce IL-6 during exercise.

Research primarily from the muscle research group in Copenhagen, Denmark has now fully established that contracting skeletal muscle is the primary source of IL-6 during exercise. Ostrowski et al. (1998) were the first to provide evidence that IL-6 is released from contracting skeletal muscle and utilised comparative polymerase chain reaction (PCR) to quantify if the vastus lateralis and/or PBMC produced IL-6 mRNA before and after a marathon. At rest, IL-6 mRNA in muscle was undetectable, but at the end of the marathon it was detectable in 4 PCR product bands (the amount of DNA was not quantified). IL-6 mRNA was not expressed in PBMC. However, in addition to myocytes, muscle biopsies can contain many other types of cells that are known to produce IL-6 including smooth muscle cells, fibroblasts, endothelial cells and macrophages (Cicco et al. 1990; De Rossi et al. 2000; Jirik et al. 1989; Klouche et al. 1999). Therefore, the increase in IL-6 mRNA detected following exercise could be from various other cells other than myocytes.

Steensberg et al. (2000) supported the finding from Ostrowski et al. (1998) and provided strong evidence that contracting skeletal muscle can account for nearly all the production and release of IL-6. Seven males performed one-legged dynamic knee extensor exercise (a purely concentric model) for 5 h at ~40% $\dot{W}_{max}$ and IL-6 release from exercising and resting legs was measured using the arterial–venous difference technique. This technique involves inserting one indwelling catheter into the femoral artery (same blood supply for both legs) and two other catheters into the femoral veins (one for each leg). The arterial–femoral venous (a–fv) differences for exercising and resting legs were determined as arterial plasma IL-6 concentration -

venous plasma IL-6 concentration. Net release of IL-6 from exercising and resting leg was calculated using Ficks principle: Blood flow (via Doppler ultrasonography) · a–fv difference. The results of this study are presented in Figure 1.22 and demonstrate that net IL-6 release from the exercising leg almost entirely accounts for the increase in the systemic plasma IL-6 concentration. Importantly, even though the femoral arteries to both the resting and exercising legs had the same supply of assorted hormones, metabolites, and other potential IL-6 mediators no net release of IL-6 was detected in the resting leg indicating that the release of IL-6 from the muscle is dependent upon the muscle contracting, and not upon secreted factors such as adrenaline. This study, however, does not conclusively demonstrate that skeletal muscle is the source of IL-6 during exercise. The a–fv difference technique can only measure the net uptake or release of a specific molecule (e.g., IL-6) over a specific area of tissue (e.g., the upper leg).

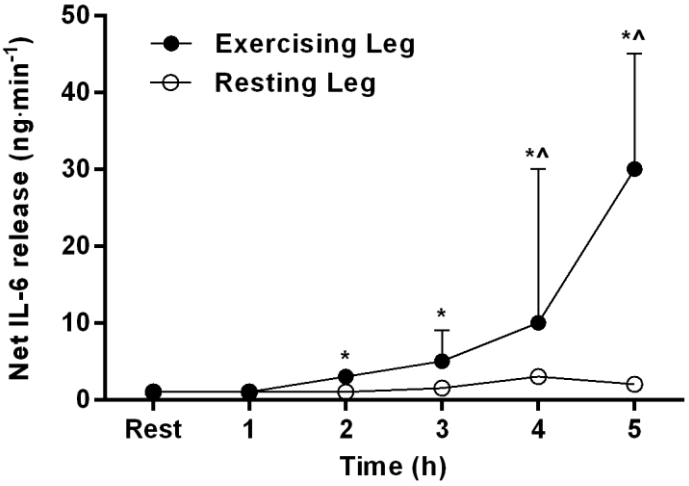


Figure 1.22 Net release of interleukin-6 (IL-6) from exercising and resting legs during 5 h one-legged dynamic knee extensor exercise at ~40% of maximal power output. Significant difference (from rest * $P < 0.05$; between exercising and resting leg $^{\wedge}$ $P < 0.05$). Values are mean ± SD. Adapted from Steensberg et al. (2000).

As discussed above, the detection of IL-6 mRNA from muscle biopsies and IL-6 production/release using the a–fv difference technique is limited. To conclusively find the cellular source of IL-6 within contracting skeletal muscle Hiscock et al. (2004) determined IL-6 in muscle protein and mRNA in myofibres before and after 2 h of semi-recumbent cycling exercise (~55% $\dot{V}O_{2peak}$) using immunohistochemistry and *in situ* hybridisation. This is a valid, reliable, and sensitive method of localising and detecting specific mRNA sequences in morphologically preserved tissue sections. After exercise, there was an increase in IL-6 protein content in the periphery of individual myofibres (Figure 1.23) and an increase in IL-6 mRNA. This finding and others (Malm et al. 2000) provide convincing evidence that contracting skeletal myocytes are the primary source of IL-6 during and following exercise. Since this finding IL-6 has been termed a myokine.

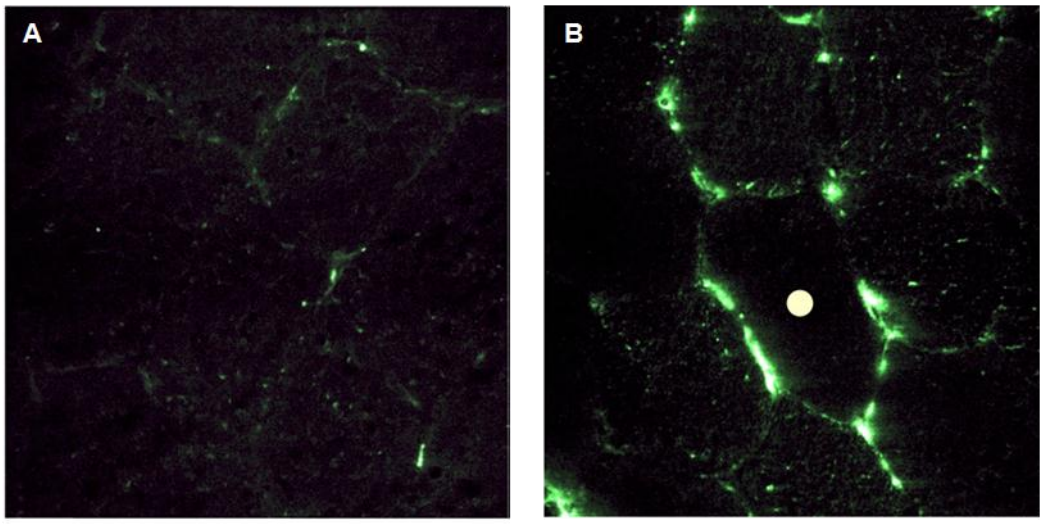


Figure 1.23 Interleukin-6 protein content in the periphery of individual human myofibres (stained green) before (A) and after (B) 2 h semi-recumbent cycling exercise at ~55% peak oxygen uptake. Adapted from Hiscock et al. (2004)

Along with myocytes, an increase in IL-6 has been detected in other sources after exercise. These include the brain (Nybo et al. 2002), adipose (Lyngso et al.

2002) and peri-tendon (Langberg et al. 2002) tissue. The mutual contribution of these sources (compared with skeletal muscle) to systemic IL-6 is unclear, but it is suggested that it they contribute very little (Pedersen and Febbraio 2008). Since almost any cell type may produce IL-6 with adequate stimulation (Akira et al. 1993), future research may reveal further sites that contribute to systemic IL-6 in response to exercise.

1.11.3 STIMULI FOR INTERLEUKIN-6 DURING MUSCULAR CONTRACTIONS

Skeletal myocytes can produce IL-6 in response to various stimuli *ex vivo*, including incubation with lipopolysaccharide (Bergmann et al. 1999), calcium-sensitive phosphatase calcineurin (Banzet et al. 2007), reactive oxygen species (ROS; Kosmidou et al. 2002), nitric oxide (NO; Makris et al. 2010), and inflammatory cytokines (Febbraio and Pedersen 2002). However, the unique characteristic of human skeletal muscle is that it can produce IL-6 during contraction in the absence of inflammatory markers (Febbraio and Pedersen 2002), and independent of TNF-α (Keller et al. 2006), linking IL-6 to metabolism rather than inflammation.

The most potent stimuli for the production of IL-6 in human skeletal muscle during exercise may be low muscle glycogen content. It is commonly understood that during prolonged and strenuous exercise the glycogen content in contracting skeletal muscle decreases and the contracting muscles become reliant upon blood glucose for an energy substrate. Based on this understanding, and the findings of Steensberg et al. (2000), it led researchers to hypothesise that IL-6 may be produced and released from the contracting muscles, signalling to the liver to increase its glucose output, maintaining substrate homeostasis (Gleeson 2000). This should prevent an extreme

fall in the blood glucose concentration. Thus, IL-6 is produced and released due to an 'energy crisis' within the contracting muscle. This hypothesis expanded on previous findings that found carbohydrate ingestion attenuates increases in plasma IL-6 concentration during prolonged and strenuous exercise (Nehlsen-Cannarella et al. 1997; Nieman et al. 1998).

To test the 'energy crisis' hypothesis Steensberg et al. (2001) employed a similar experimental design to the study undertaken previously (Steensberg et al. 2000), but with pre-exercise muscle glycogen stores altered. The day before the main experimental trial, muscle glycogen content in the quadriceps of one leg was depleted by participants undertaking 1 h of one-legged cycling exercise followed by 1 h of two-armed cranking. On the day of the main experimental trial, participants then performed 4–5 h (point of volitional tolerance) of two-legged knee extensor exercise at ~40% $\dot{W}_{max}$. Once again, the a–fv difference technique was utilised. Figure 1.24 clearly shows that during the first 3 h of exercise the net release of IL-6 was higher in the glycogen depleted leg than the control leg, but they were similar post exercise. These results are supported by others (Keller et al. 2001) and provide strong evidence that the glycogen availability in contracting skeletal muscle is associated with changes to the rate of IL-6 production and release in those muscles during exercise - supporting the 'energy crisis' hypothesis.

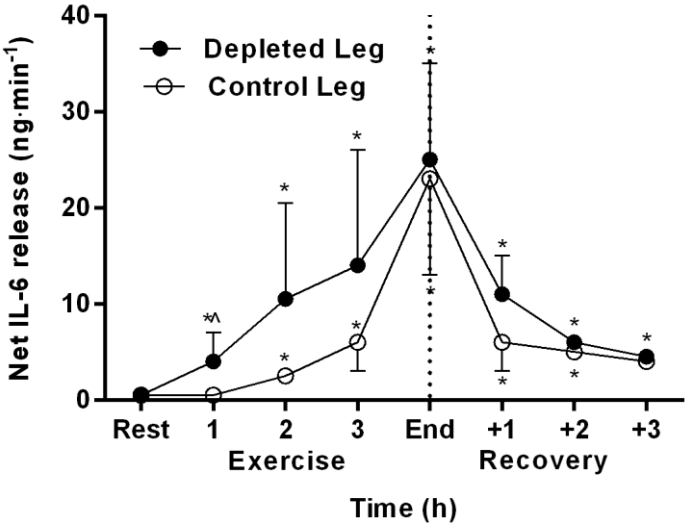


Figure 1.24 Net release of interleukin-6 (IL-6) from glycogen depleted and control legs during and in recovery from 4−5 h one-legged dynamic knee extensor exercise at ~40% of maximal power output. Significant difference (from rest $^{*}P < 0.05$; between glycogen depleted and control leg $^{\wedge}$ $P < 0.05$). Values are mean ± SD. Adapted from Steensberg et al. (2001).

1.11.4 POSSIBLE BIOLOGICAL ROLES OF INTERLEUKIN-6 DURING MUSCULAR CONTRACTIONS

The pleiotropic nature of IL-6 means that it has a variety of biological roles during muscular contractions, and when it is released peripherally it acts in an endocrine like manner (Table 1.4 and Figure 1.25). Febbraio et al. (2004) have elegantly shown that IL-6 released from contracting skeletal muscle during exercise may signal to the liver to stimulate hepatic glucose production. Participants performed 2 h of cycling exercise on separate days at 70% $\dot{V}O_{2peak}$, 40% $\dot{V}O_{2peak}$ or 40% $\dot{V}O_{2peak}$ plus constant infusion of recombinant human (rh) IL-6 at a rate intended to match the elevated plasma IL-6 concentration observed during the 70% $\dot{V}O_{2peak}$ trial (~9 pg·mL^{-1}). Hepatic glucose production was estimated from endogenous glucose production by infusing participants with a glucose stable isotope during all trials. Endogenous glucose production was significantly higher during the 40% $\dot{V}O_{2peak}$ plus rhIL-6 trial compared to the 40% $\dot{V}O_{2peak}$ trial, and was very similar to

the endogenous production seen during the 70% $\dot{V}O_{2peak}$ trial. Interestingly, there were no differences in the systemic concentrations of cortisol, insulin, glucagon or catecholamines (adrenaline or noradrenaline) between 40% $\dot{V}O_{2peak}$ plus rhIL-6 trial compared to the 40% $\dot{V}O_{2peak}$ trial suggesting that IL-6 was responsible for stimulating hepatic glucose production and not these markers that are also known to stimulate glucose production.

Table 1.4 Possible biological roles of interleukin-6 during muscular contraction.

Reference	Possible biological role
Carey et al. (2006)	Stimulate glucose uptake in myocytes
Febbraio et al. (2004)	Stimulate hepatic glucose production
Steensberg et al. (2003)	Stimulate C-reactive protein, cortisol, IL-1ra and IL-10 production
Starkie et al. (2003)	Inhibit TNF-α production
Wolsk et al. (2010)	Stimulate whole-body lipolysis and fat oxidation
Wolsk et al. (2010)	Stimulate skeletal muscle lipolysis
Nemet et al. (2006)	Stimulate growth hormone and inhibit IGF-1 production

IL-1ra, interleukin-1 receptor antagonist; IL-10, interleukin-10; TNF-α, tumor necrosis factor-α; IGF-1, insulin-like growth factor-1.

Carey et al. (2006) have reported an increase in glucose uptake in L6 myotubes incubated with IL-6 via an increase in translocation of glucose transporter type 4 (GLUT4) from intracellular pools to the plasma membrane. This occurred through the phosphatidylinositol 3-kinase (PI3-K) pathway (Figure 1.25). This finding suggests that within skeletal muscle IL-6 may stimulate glucose uptake in myocytes, but whether this effect occurs *in vivo* in exercising humans remains unknown.

It has been demonstrated that IL-6 has a peripheral biological role in stimulating lipolysis and fat oxidation using human rhIL-6 infusion for whole-body

(Petersen et al. 2005; van Hall et al. 2003) or subcutaneous adipose tissue (Lyngso et al. 2002) measurements. Recently, Wolsk et al. (2010) have provided simultaneous recordings of whole-body, subcutaneous adipose tissue and skeletal muscle measurements of lipolysis and fat oxidation in healthy humans. Lipolysis was estimated by infusing participants with a fatty acid stable isotope, and fat oxidation by measuring expired gases to calculate the respiratory exchange ratio. Catheters were placed into skeletal muscle and adipose tissue to record blood measurements. Infusing rhIL-6 for 4 h at a rate that ended in a plasma concentration of 42.19 ± 2.70 $pg \cdot mL^{-1}$ (similar to a marathon) resulted in an increase in whole-body lipolysis and fat oxidation, skeletal muscle lipolysis, but no effect on adipose tissue lipolysis. There were also no differences in systemic concentrations of insulin, glucagon, or adrenaline (substances that are known to stimulate lipolysis). There was a slight increase in cortisol, but its actions on stimulating lipolysis are still unclear (Macfarlane et al. 2008). Interestingly, using this *in vivo* model with humans, Wolsk et al. (2010) did not confirm that lipolysis and fatty acid oxidation occurs via an increase in the phosphorylation of AMP-activated protein kinase (AMPK) and signal transducer and activator of transcription 3 (STAT3) within skeletal muscle. This has been previously reported in rat skeletal muscle (Kelly et al. 2009a), L6 myotubes (Petersen et al. 2005) and human myocytes (Al-Khalili et al. 2006). Wolsk et al. (2010) found that STAT3 was only involved. The difference may be explained by the dose of IL-6 used for *in vitro* experiments where concentrations are typically 1000-fold higher than those seen physiologically in humans. Figure 1.25 has, therefore, been adapted with this new information from the figure originally presented by Pedersen and Febbraio (2008).

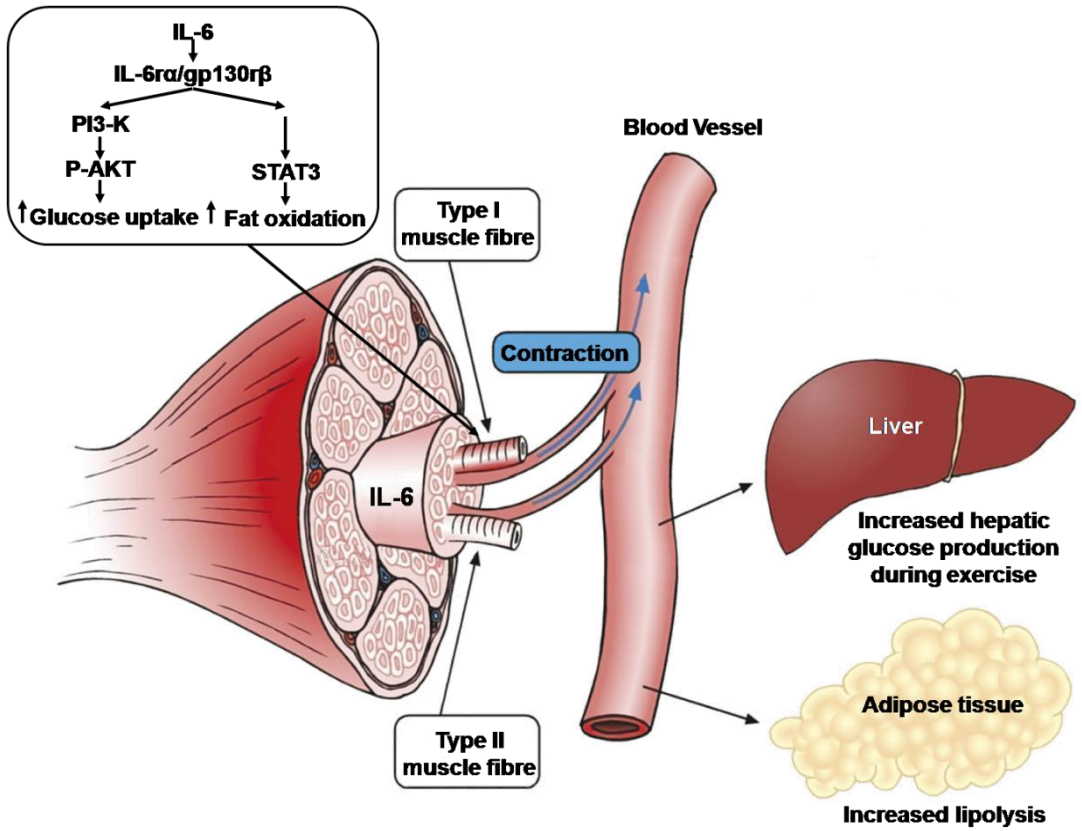


Figure 1.25 Possible biological roles of interleukin-6 (IL-6) during muscular contractions. Type I and type II muscle fibres produce IL-6 which subsequently exerts its effects both locally within the muscle, and when released into the circulation peripherally in several organs in an endocrine like manner. In skeletal muscle IL-6 acts in an autocrine or paracrine manner to signal through a glycoprotein 130 homodimer and ligand binding chain (gp130rα/IL-rβ) to result in activation of phosphatidylinositol 3-kinase (PI3-K) or signal transducer and activator of transcription 3 (STAT3) to increase glucose uptake or fat oxidation. Adapted from Pedersen and Febbraio (2008).

IL-6 possesses both pro- and anti-inflammatory properties, but the current view is that it is primarily an anti-inflammatory cytokine (Petersen and Pedersen 2006). When IL-6 is infused into humans it results in shock, but unlike classic cytokines TNF-α and IL-1, it does not cause fever and capillary leakage syndrome (Tilg et al. 1997). Therefore, IL-6 seems to have a role in inducing hepatocyte derived acute phase proteins (e.g., C-reactive protein) of which many have anti-inflammatory properties (Steensberg et al. 2003). The anti-inflammatory properties of IL-6 also include the direct inhibition of the pro-inflammatory cytokine TNF-α

(Starkie et al. 2003) and the upregulation of cortisol and anti-inflammatory cytokines IL-1ra and IL-10 (Steensberg et al. 2003). It has been suggested that IL-6 mediates cortisols effects on neutrophil binding (Steensberg et al. 2003). IL-1ra prevents signal transduction of IL-1 by blocking IL-1 receptors (Dinarello 2000) and IL-10 is a potent inhibitor of Th1- monocyte- and macrophage- derived cytokines and attenuates the surface expression of TNF-α receptors amongst other roles (Dickensheets et al. 1997; Joyce et al. 1994).

It has been reported that when very high concentrations of rhIL-6 are infused (peak concentration of 106.2 ± 9.6 $pg \cdot mL^{-1}$) for 3 h into healthy humans there are increases in growth hormone and decreases in insulin-like growth factor-1 (Nemet et al. 2006). However, when lower concentrations of rhIL-6 are infused this effect is not seen (Febbraio et al. 2004) suggesting that there may be an optimum concentration required to achieve this effect.

1.12 OTHER CYTOKINES IN EXERCISE

Although other cytokines are observed in the systemic circulation following prolonged and strenuous exercise (Figure 1.19) very little is known about their cellular sources and/or biological roles. Table 1.5 reports the findings of Chan et al. (2004) who have shown that the mRNA expression of a number of cytokines and chemokines are detectable in skeletal muscle at rest, but after cycling exercise (1 h at 70% $\dot{V}O_{2peak}$) with and without prior glycogen depletion there was only an increase in the mRNA expression of IL-6 and IL-8. The results of this study are complimented by Nieman et al. (2003) who also reported the same findings before and after treadmill running (3 h at 70% $\dot{V}O_{2peak}$) with and without carbohydrate feeding. Both studies suggest that IL-8 may have a biological role similar to IL-6.

Nieman et al. (2003), however, also reported that IL-1β and TNF-α were detectable after exercise without carbohydrate feeding. It is probable that this response was due to an inflammatory response from muscle damage and/or injury occurring during running exercise. During cycling, a concentric exercise, this may not occur. The possible biological role for these cytokines during muscle damage and/or injury will be explained later (see BIOLOGICAL ROLES OF BREATHING-INDUCED CYTOKINES).

Table 1.5 Detection of cytokine mRNA expression at rest and after 1 h cycling exercise at 70% peak oxygen uptake with and without prior glycogen depletion. Adapted from Chan et al. (2004).

Cytokine	Expression		Influenced by glycogen content
	Rest	Exercise	
IL-1α	No	No	No
IL-1β	Yes	No	No
IL-2	No	No	No
IL-4	No	No	No
IL-5	No	No	No
IL-6	Yes	Yes	Yes
IL-8	Yes	Yes	Yes
IL-10	No	No	No
IL-12p35	No	No	No
IL-12p40	No	No	No
IL-15	Yes	No	No
IFN-γ	No	No	No
TNF-α	Yes	No	No

IL-1α, interleukin-1α; IL-1β, interleukin-1β; IL-2, interleukin-2; IL-4, interleukin-4; IL-5, interleukin-5; IL-6, interleukin-6; IL-8, interleukin-8; IL-10, interleukin-10; IL-12p35, interleukin-12p35; IL-12p40, interleukin-12p40; IL-15, interleukin-15; IFN-γ, interferon-γ; TNF-α, tumor necrosis factor-α.

Large increases in the plasma concentrations of IL-1ra and IL-10 are observed following prolonged and strenuous exercise (Figure 1.19). Nieman and colleagues (Nieman et al. 2006; Nieman et al. 2007) reported that after 2 h cycling exercise at ~ 64% $\dot{W}_{max}$ IL-1ra and IL-10 mRNA expression in blood leukocytes are increased. Furthermore, there was also an increase in IL-8, but not IL-6 mRNA

expression in leucocytes suggesting that IL-8 may have dual cellular sources, and supports the evidence that IL-6 is primarily released from skeletal muscle.

1.13 BREATHING-INDUCED CYTOKINES

1.13.1 BREATHING-INDUCED SYSTEMIC CYTOKINE RESPONSE

A series of excellent studies by Vassilakopoulos and colleagues have provided compelling evidence that the respiratory muscles can produce and release cytokines (Sigala et al. 2011; Sigala et al. 2012; Vassilakopoulos et al. 1999; Vassilakopoulos et al. 2002; Vassilakopoulos et al. 2004). Vassilakopoulos et al. (1999) reported increases in the plasma concentrations of IL-6 and IL-1β of up to 130% after IRL at ~75% MIP until task failure which occurred after 54 ± 10 min. There was no significant increase in the plasma concentrations of these cytokines after IRL at ~35% MIP for the same duration suggesting that this was not an effect of experimental conditions. A later study (Vassilakopoulos et al. 2002) confirmed these observations using the same IRL protocol for 45 min and also reported an increase in plasma TNF-α concentration (Figure 1.26). Using intracellular flow cytometry, Vassilakopoulos et al. (2002) also excluded monocytes as the cellular source of the cytokines. Thus, the increase in plasma cytokine concentration was attributed to increased rates of release from the inspiratory muscles.

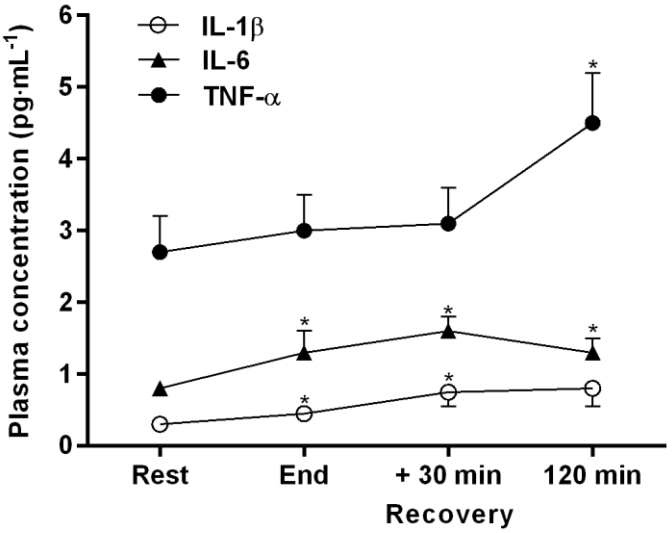


Figure 1.26 Plasma concentration of interleukin-1β (IL-1β), interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) at rest and in recovery from 45 min inspiratory flow-resistive loading at ~75% maximal inspiratory pressure. Significant difference (from rest * $P < 0.05$). Values are mean ± SD. Adapted from Vassilakopoulos et al. (2002).

1.13.2 SOURCE OF BREATHING-INDUCED SYSTEMIC CYTOKINE RESPONSE

Sigala et al. (2011) supported this suggestion and reported that when anaesthetised rats were exposed to IRL (~48% peak tracheal pressure) for either 1, 3 or 6 h, or quietly breathed (control) for 6 h there was a significant time-dependent increase in the diaphragm protein expression of IL-6, and to a lesser extent IL-1β, TNF-α and IL-10 (Figure 1.27). These findings confirmed earlier reports of a significant increase in cytokine mRNA expression in the diaphragms of rats after a similar IRL protocol (Vassilakopoulos et al. 2004).

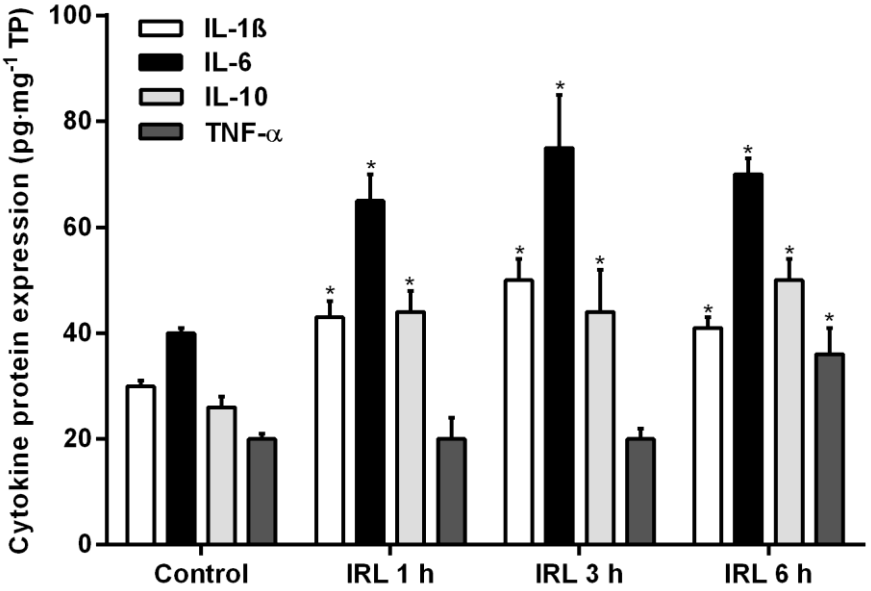


Figure 1.27 Protein expression of cytokines interleukin-1β (IL-1β), interleukin-6 (IL-6), interleukin-10 (IL-10) and tumor necrosis factor-α (TNF-α) in the diaphragm of rats after 6 h of tidal breathing (control) or 1, 3 and 6 h of inspiratory flow-resistive loading at ~48% peak tracheal pressure. Significant difference (from control * $P < 0.05$). Values are mean ± SE. Adapted from Sigala et al. (2011).

More evidence for the upregulation of cytokines within the respiratory muscles due to increased respiratory muscle work has come from patients with stable COPD. The inspiratory muscles of COPD patients experience increased workloads due to airflow limitation, hyperinflation, and a reduction in inspiratory muscle contractile properties (Casaburi 2001; Levine et al. 2003). Casadevall et al. (2007) reported that the external intercostals (taken via biopsy) of COPD patients had higher mRNA and protein expression of TNF-α, IL-1β and IL-6 (although IL-6 mRNA was not significant) compared to aged-matched healthy controls. There were no differences for IL-10. MIP in COPD patients was 65 ± 22% of predicted values and they also had lower inspiratory muscle endurance compared to healthy controls. Whether the upregulation of respiratory muscle cytokines in COPD patients spills out into the systemic circulation and contributes to the systemic inflammation observed (Garcia-Rio et al. 2010) is unknown. It could be speculated, however, that they do as

the plasma response of TNF-α from COPD patients during moderate intensity exercise is greater than the response observed in healthy controls (Rabinovich et al. 2003). The source of this increase could not be determined as TNF-α mRNA in the vastus lateralis was unchanged in both groups after exercise.

There may be other sources of the systemic cytokines observed after IRL in healthy humans (Vassilakopoulos et al. 1999; Vassilakopoulos et al. 2002). Cytokines can be upregulated within the lungs of murines exposed to negative or positive pressure induced hyperventilation (von Bethmann et al. 1998) or IRL (Toumpanakis et al. 2010) and it could be speculated these could enter the systemic circulation from permeation of epithelial and endothelial barriers with increased transmural pressures (Uhlig 2002). However, these results were obtained by profound overdistention of the lung, which is unlikely during IRL in humans, since they can self-regulate end-inspiratory and/or end-expiratory lung volumes.

1.13.3 BIOLOGICAL ROLES OF BREATHING-INDUCED CYTOKINES

The biological roles of breathing-induced cytokines during strenuous respiratory muscle contractions in humans are unknown. It is probable that IL-6 has the same roles in metabolism observed in whole-body exercise (Figure 1.28). This notion would be supported by the reductions in diaphragm glycogen content of murines exposed to IRL (Ciufo et al. 2001) and whole-body exercise (Green et al. 1987; Green et al. 1988; Ianuzzo et al. 1987; Namiot et al. 1985; Namiot and Gorski 1988). IL-1β and TNF-α may have a role in mediating muscle damage and/or injury and evidence also suggests they may compromise diaphragmatic contractility (Figure 1.28).

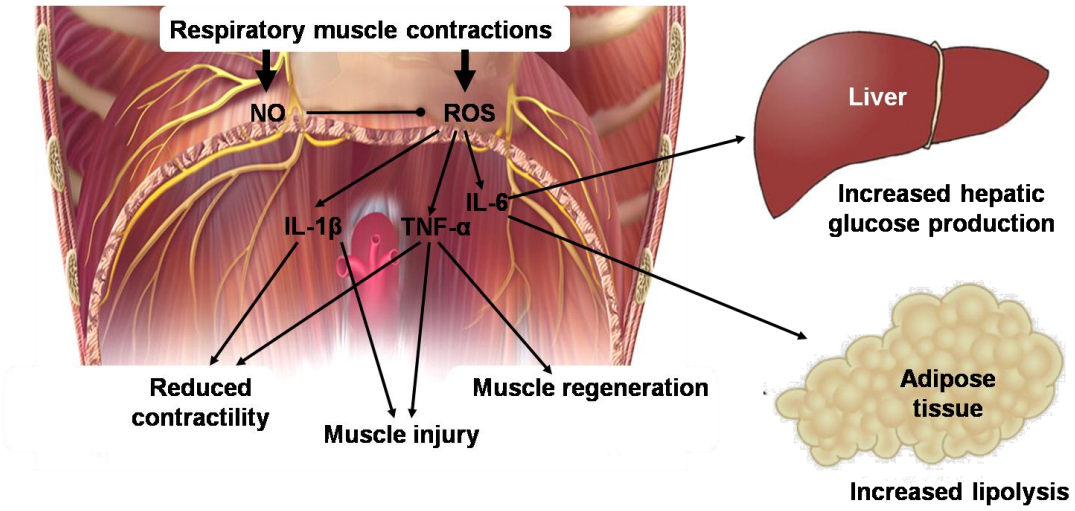


Figure 1.28 Possible biological roles of breathing-induced cytokines interleukin-1β (IL-1β), interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) during strenuous respiratory muscle contractions. Strenuous respiratory muscle contractions may augment reactive oxygen species (ROS) which are mediated by nitric oxide (NO) and subsequently upregulate IL-6, IL-1β and TNF-α. When IL-6 is released into the peripheral circulation it exerts its effects on several organs in an endocrine like manner. Within the muscle, IL-1β and TNF-α may both reduce muscle contractility and mediate muscle injury. TNF-α may also mediate muscle regeneration. Adapted from Vassilakopoulos and Hussain (2007).

1.13.3.1 MUSCLE DAMAGE

Significant diaphragm injury has been observed in animals after exposure to severe resistive loads (Jiang et al. 1998; Jiang et al. 2001; Palacio et al. 2002; Reid and Belcastro 2000; Simpson et al. 2004; Wang et al. 2005; Zhu et al. 1997), after IRL in humans (Foster et al. 2012; Orozco-Levi et al. 2001; Palacio et al. 2002) and in COPD patients at rest (Casadevall et al. 2007; Casadevall et al. 2009). Many factors may be involved in this process including increased activity of the proteolytic enzyme caplain or augmented ROS production (Jiang et al. 2001; Sigala et al. 2011).

IL-1β may also contribute to the development of muscle injury through the upregulation of adhesion molecule expression on the surface of endothelial cells, leading to augmentation of transendothelial migration of blood-borne inflammatory markers (Cannon et al. 1989). This response results in the initial recruitment of

neutrophils, and later macrophages, into muscle interstitial sites. When rabbits were exposed to 1.5 h of IRL there was significant fibre injury detected in the diaphragms 72 h later (Wang et al. 2005). This was associated with significant upregulation of intracellular adhesion molecule-1 expression in blood vessels that traversed the diaphragm and a large infiltration of macrophages and neutrophils in the interstitium of the diaphragm. A similar response of muscle injury followed by increased intramuscular IL-1β expression has been observed following whole-body eccentric exercise in humans (Fielding et al. 1993).

Although experimental data for the role of TNF-α in muscle injury is lacking it might also contribute by augmenting oxidative stress (see OXIDATIVE STRESS) in a paracrine fashion (Li and Reid 2001). Increased oxidative stress has been observed in the diaphragm up to 3 days after severe resistive loading in rabbits, and the change could not be attributed to an acute increase in cytokine production (Jiang et al. 2001).

It must be noted that the increase in pro-inflammatory cytokines involved in muscle damage is limited by the compensatory upregulation of anti-inflammatory cytokines IL-4 and IL-10 (Sigala et al. 2011; Sigala et al. 2012; Vassilakopoulos et al. 2004) which minimise the harmful biological effects of pro-inflammatory cytokines.

1.13.3.2 MUSCLE REGENERATION

Although several reports have implicated pro-inflammatory cytokines in the development of muscle injury there is evidence that they may also be involved in muscle regeneration. For example, TNF-α and IL-1β are upregulated in skeletal muscle several days after the development of muscle injury from whole-body exercise and they may act to enhance proteolytic removal of damaged proteins and

cells through the recruitment and activation of phagocytes (Paulsen et al. 2012). *In vitro* experiments using double knockout mice have demonstrated that when TNF-α synthesis is blocked the repair of damaged muscle is prevented (Chen et al. 2005; Chen et al. 2007). In COPD patients expression of TNF-α in the external intercostals was directly related to both general muscle damage, and a direct relationship was observed between markers of muscle damage and markers of regeneration (Casadevall et al. 2009). Taken together, these reports suggest that once the muscle is damaged TNF-α acts to help repair and regenerate the muscle.

1.13.3.3 MUSCLE CONTRACTILITY

In addition to the effect of pro-inflammatory cytokines on muscle injury and regeneration, TNF-α may also be involved in inspiratory muscle contractility. Wilcox et al. (1994) reported that when anaesthetised, mechanically ventilated dogs were infused with TNF-α for up to 6 h, there was a significant decrease in the P_{di} response to bilateral electrical phrenic nerve stimulation at a range of frequencies; demonstrating significant diaphragm fatigue (Figure 1.29). There was no effect on P_{di} in control dogs receiving saline infusion. Several other reports support a role for TNF-α in reducing respiratory muscle contractility. *In vitro* experiments on isolated diaphragm strips have shown reduced diaphragm contractility after incubation with TNF-α (Hardin et al. 2008; Reid et al. 2002; Wilcox et al. 1996). An increased expression of TNF-α in the external intercostals of COPD patients is correlated with reduced MIP and inspiratory muscle endurance. However, it must be noted that the supraphysiological dose of TNF-α infused into dogs (Wilcox et al. 1994), or incubated with isolated diaphragm strips (Hardin et al. 2008; Reid et al. 2002; Wilcox et al. 1996) would not be seen in humans undertaking IRL or whole-body

exercise. Significant increases in the protein expression of TNF-α only occur after 6 h of IRL in rats suggesting significant muscle damage is required to elicit a response (Figure 1.27; Sigala et al. 2011). Furthermore, the increased expression of TNF-α in the external intercostals of COPD patients (Casadevall et al. 2007; Casadevall et al. 2009) are due to several years of muscle dysfunction.

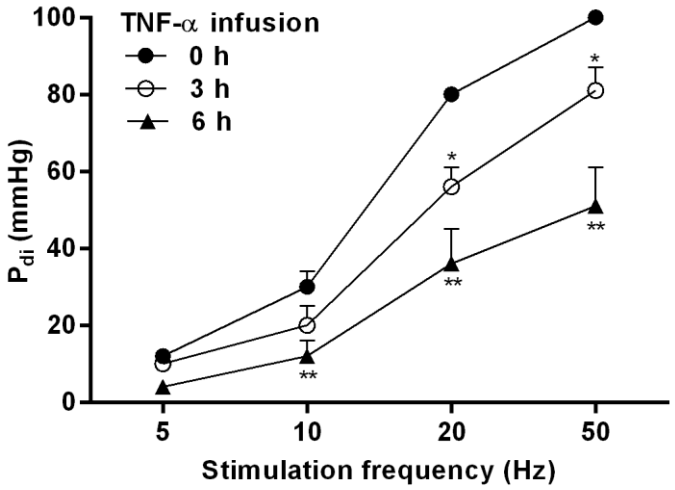


Figure 1.29 Transdiaphragmatic pressure (P_{di}) response to supramaximal bilateral electrical phrenic nerve stimulation at a range of frequencies after 0, 3 and 6 h of tumor necrosis factor-α (TNF-α) infusion in dogs. Significant difference (from 0 h * $P < 0.05$, ** $P < 0.01$). Values are mean ± SE. Adapted from Wilcox et al. (1994).

1.14 OXIDATIVE STRESS

It has been recently demonstrated that breathing-induced cytokines are upregulated by oxidative stress. At rest there is a continuous production of free radicals (atoms, molecules, ions with unpaired electrons or an open shell). The antioxidant defence mechanisms remove most free radicals before they can damage biomolecules such as lipids, proteins and nucleic acids. In resting healthy humans these processes are in balance so that a steady-state exists; there is always a certain amount of free radicals with a corresponding level of oxidative damage. Oxidative

stress refers to a state where there is either an excessive production of free radicals, or deficient antioxidant defences (Sies 1985).

1.14.1 FREE RADICAL FORMATION

It is generally considered that free radicals and, in particular, ROS are formed during oxygen metabolism in the mitochondria (Di Meo 2001). The process of ATP production through oxidative phosphorylation involves the transport of hydrogen ions across the inner mitochondrial membrane through the electron transport chain. Within the electron transport chain, electrons are passed along a series of proteins via oxidation-reduction reactions. Each acceptor protein along the chain has a greater reduction potential than the previous and an oxygen molecule is the final destination for an electron along the chain. The oxygen is normally reduced to water, but in about 0.1−2% of electrons passing along the chain, the oxygen is incompletely reduced to produce a superoxide radical (Di Meo 2001).

1.14.2 ANTIOXIDANT DEFENCE MECHANISMS

A well organised system of both enzymatic and nonenzymatic antioxidants within muscle fibres protects against oxidative stress. These antioxidants are located throughout the cytoplasm and in various organelles (e.g., mitochondria). Both enzymatic and nonenzymatic antioxidants are also located within the extracellular and vascular space. The primary enzymatic antioxidants include catalase, glutathione peroxidise and superoxide dismutase and are accompanied by peroxiredoxin, glutaredoxin and thioredoxin (Powers and Jackson 2008). These enzymatic antioxidants are complimented by numerous nonenzymatic antioxidants including bilirubin, glutathione and uric acid and by dietary antioxidants including vitamin C, vitamin E and carotenoids (Powers and Jackson 2008).

1.14.3 MEASUREMENT OF OXIDATIVE STRESS

The measurement of oxidative stress *in vivo* is difficult as ROS and reactive nitrogen species are highly reactive and/or have a very short half-life (< 1 s for some) (Palmieri and Sblendorio 2007). The measurement can be estimated from 1) free radicals; 2) radical mediated damages to lipids, proteins and nucleic acids; and 3) antioxidant enzyme activity or concentration (Palmieri and Sblendorio 2007). A complete review of the topic is beyond the scope of this thesis. Therefore, only a brief overview will be given.

Free radical production can be measured using direct methods. Electron spin resonance is a direct spectroscopic technique that allows direct measurement of ROS from their paramagnetic properties (Palmieri and Sblendorio 2007). However, the *in vivo* technique cannot be used in humans because of the toxicity of the measurement (Palmieri and Sblendorio 2007).

A basic measurement of oxidative damage is the analysis of the rate of peroxidation of membrane lipids or fatty acids. Lipid peroxidation results in the breakdown of lipids to an array of primary oxidation products such as conjugated dienes or lipid hydroperoxides and secondary oxidation products such as malondialdehyde, F2-isoprostane or expired pentane, ethane or hexane (Palmieri and Sblendorio 2007). Malondialdehyde is produced during fatty acid auto-oxidation and is most commonly measured by its reaction with thiobarbituric acid which generates thiobarbituric acid reactive substances (TBARS). Although TBARS is accepted as a general marker of lipid peroxidation, it is not specific to malondialdehyde and can overestimate malondialdehyde (Palmieri and Sblendorio 2007). F2-isoprostanes are produced by free radical catalysed peroxidation of arachidonic acid (Roberts 2000). Many recent studies have shown that F2-isoprostanes are a reliable method for

endogenous lipid peroxidation (Roberts 2000). Another lipid peroxidation technique is the measurement of volatile hydrocarbon end products in expired air such as pentane, hexane and ethane. This method has the strength of being non-invasive, but as these gases can be formed by other ways than free radical oxidation it is limited by a lack of precision (Rimbach et al. 1999).

Free radical modification of proteins causes formation of carbonyl groups into amino acid side chains (Stadtman and Levine 2000). The most common analysis of free radical damage to proteins is the measurement of carbonyl formation (Stadtman and Levine 2000). Total proteins are often also measured to give the carbonyl/protein ratio which increases the precision of protein oxidation (Chen et al. 2001). This method is of interest because the half-life of the carbonyl is long and a high level of carbonyl can show the cumulative effects of oxidative stress (Chen et al. 2001).

Free radicals can induce several types of damage to nucleic acids including DNA strand and protein cross link breaks and base modifications. There are various methods to assess free radical damage to nucleic acids (Dizdaroglu et al. 2002). One method is the modified comet assay which can measure oxidative damage with the use of bacterial repair endonucleases with appropriate specificities. The comet assay is a rapid, sensitive and reliable measurement of systemic oxidative stress in cells such as PBMC (Collins 2009; Singh et al. 1988). Comparison of findings is confounded, however, by the variety of protocols utilised by different laboratories.

Antioxidant activity can be measured in enzymatic enzymes (catalase, glutathione peroxidise and superoxide dismutase), nonenzymatic antioxidants (bilirubin, glutathione and uric acid) and by numerous dietary antioxidants (vitamin C, vitamin E and carotenoids acid) (Palmieri and Sblendorio 2007). Antioxidants are

typically measured in blood or urine and the measurement techniques are simple and reliable. However, changes in antioxidant status during acute and chronic exercise may represent a redistribution of antioxidants between tissue and plasma (Ji 1995).

In summary, all the methods discussed have limitations and because of the complexity of oxidative stress no single measurement will summarise all the effects. If the laboratory is not limited by expense or time a battery of markers would be the most beneficial (Palmieri and Sblendorio 2007). If, however, only one method can be utilised it should asses the effects of oxidative stress on eithier a specific site of interest, or be reliable at measureing stystemic oxidative stress (Palmieri and Sblendorio 2007).

1.14.4 BREATHING-INDUCED CYTOKINES AND OXIDATIVE STRESS

When rats were exposed to 6 h of IRL and pre-treated with ROS inhibitors there was a decrease in diaphragmatic cytokines via P38 mitogen activated protein kinase (P38 MAPK) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathways (Sigala et al. 2011). NO regulates NF-κB and MAPK activity and, therefore, when rats were pre-treated with NO inhibitors and exposed to 6 h of IRL there was an increase in diaphragmatic cytokines (Sigala et al. 2012). Taken together this research suggests that ROS regulates cytokine production within the diaphragm and NO mediates this production (Figure 1.28). However, it must be noted that although hypoxemia was prevented in rats exposed to IRL by supplementing the inspiratory line with 100% O_2, they still experienced significant respiratory acidosis. Arterial pH in rats exposed to IRL significantly decreased from 7.4 ± 0.01 at rest to 7.0 ± 0.02 after 6 h (Sigala et al. 2011). Since oxidative stress can be upregulated by respiratory acidosis (Arbogast and Reid 2004) it is difficult to

determine whether the increase in oxidative stress within the diaphragm was due to strenuous contractions, or respiratory acidosis.

The observation that oxidative stress upregulates breathing-induced cytokines in rats has also been supported in humans. When participants were supplemented with a combination of antioxidants (to scavenge ROS) and undertook IRL (45 min at ~75% MIP) the plasma concentration of IL-1β was undetectable, there was no increase in the plasma concentration of TNF-α, and the increase in IL-6 was significantly reduced (Vassilakopoulos et al. 2002). Furthermore, supplementation of antioxidants (vitamin C and E to reduce ROS), or infusion with nitro-L-arginine methyl (to increase NO) both reduce IL-6 production from skeletal muscle during leg extension exercise (Fischer et al. 2004a; Steensberg et al. 2007).

1.14.5 BREATHING-INDUCED OXIDATIVE STRESS

Many reports have conclusively shown that oxidative stress is increased in the respiratory muscles of animals following exposure to severe resistive loads (Anzueto et al. 1992; Barreiro et al. 2006; Borzone et al. 1994; Ciufo et al. 1995; Goodyear-Bruch et al. 2008; Jiang et al. 2001; Sigala et al. 2011; Sigala et al. 2012; Vassilakopoulos et al. 2007), endurance exercise (Itoh et al. 2004; Lawler et al. 1994; Oh-ishi et al. 1997; Powers et al. 1994b; Vincent et al. 1999; Vincent et al. 2000) and *in vitro* fatiguing stimulation of isolated diaphragm fibres (Diaz et al. 1993; Kolbeck et al. 1997; Reid et al. 1992; Reid et al. 2002).

Furthermore, compared to healthy human controls, levels of oxidative stress are higher in the respiratory muscles of COPD patients (Barreiro et al. 2005; Marin-Corral et al. 2009; Wijnhoven et al. 2006). Recently, using immunoblotting and histochemical staining techniques, Marin-Corral et al. (2009) reported increased

oxidation of proteins involved in energy production and contractile performance (creatine kinase, carbonic anhydrase III, actin and myosin) and increased superoxide anion in the diaphragm (taken with a biopsy) of severe COPD patients. This increase in oxidative stress is a likely contributor to the respiratory muscle dysfunction (decreased strength and endurance) observed in these patients.

1.14.6 BREATHING-INDUCED OXIDATIVE STRESS AND RESPIRATORY MUSCLE FATIGUE

Reports have demonstrated that respiratory muscle contractile properties are altered by oxidative stress. A decrease in muscle contractility has been observed *in vitro* by incubating isolated muscle fibres with ROS (Lawler et al. 1997) and NO (Lawler and Hu 2000). Conversely, contractile performance is improved by administrating antioxidants before or during fatiguing contractions *in vitro* (Diaz et al. 1994; Khawli and Reid 1994; Shindoh et al. 1992) and *in vivo* (Kelly et al. 2009b; Supinski et al. 1995; Supinski et al. 1997; Travaline et al. 1997b). For example, time to volitional tolerance during IRL (~80 P_{dipeak}) was increased from 14 to 23 min following intravenous infusion of the antioxidant N-acetylcysteine compared to placebo infusion and moreover, the P_{ditw} response to bilateral electrical phrenic nerve stimulation was significantly reduced up to 24 h after task failure (Figure 1.30; Travaline et al. 1997b). This study indicates that by increasing the diaphragms antioxidant defence mechanisms against oxidative stress inspiratory muscle endurance can be increased, and diaphragm fatigue reduced.

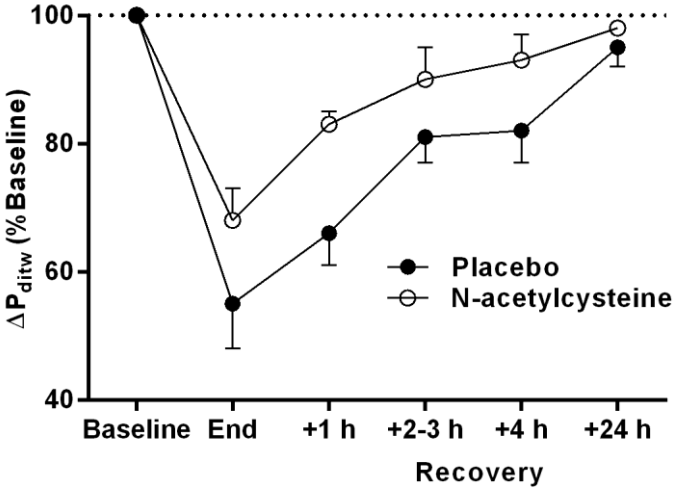


Figure 1.30 Transdiaphragmatic twitch pressure (P_{ditw}) response to supramaximal bilateral electrical phrenic nerve stimulation before and in recovery from inspiratory resistive loading at ~80% peak transdiaphragmatic pressure with N-acetylcysteine or placebo infusion in healthy humans. Values are mean ± SD. Adapted from Travaline et al. (1997b).

1.15 EFFECTS OF TRAINING ON CYTOKINES AND OXIDATIVE STRESS

1.15.1 TRAINING AND CYTOKINES

Compared to healthy counterparts, chronic heart failure patients have reduced inspiratory muscle strength and endurance (Ribeiro et al. 2009) and higher systemic cytokine concentrations at rest (Orus et al. 2000). It could, therefore, be speculated that these higher contractile demands result in an upregulation of respiratory muscle cytokines that spill out into the systemic circulation, and that RMT would attenuate this. Nonetheless, serum concentrations of IL-6 and TNF-α do not change after 10 weeks of flow-resistive loading (3 times a week at ~60% MIP) (Laoutaris et al. 2007). It cannot be determined from this study whether the local expression of cytokines within the respiratory muscles changed after training.

At the end of 90 min treadmill running exercise IL-1 was lower in the diaphragms of rats that undertook prior long-term endurance exercise training (up to 90 $min \cdot d^{-1}$ for 9 weeks) than untrained controls (Oh-ishi et al. 1997). This suggests

that IL-1 may be reduced in the respiratory muscles after exercise training. However, IL-1 was measured with the thymocyte proliferation assay which was developed before the availability of recombinant IL-1 proteins, and the assay can detect both IL-1 and IL-6 (Bagby et al. 1996). Therefore, it may be that it was IL-6, and not IL-1, that was reduced after exercise training. This would be supported by the reduction in IL-6 within lower limb muscle of humans following endurance exercise training (Fischer et al. 2004b; Yfanti et al. 2012).

Fischer et al. (2004b) were the first to report the effect of exercise training on IL-6 mRNA expression in the vastus lateralis of healthy humans. Before and after dynamic one legged knee extension training (5 $d \cdot wk^{-1}$ at ~75% $\dot{W}_{max}$ for 10 weeks) participants performed a 3 h acute bout of the same exercise at 50% of their pre-training $\dot{W}_{max}$ (the same relative power). The absolute power was 44% higher after training. The increase in IL-6 mRNA expression after the acute exercise was 76-fold before training, but only 8-fold after training. Furthermore, resting skeletal muscle glycogen content had increased by ~74% after training and the resting muscle glycogen levels correlated negatively ($r = -0.34$, $P < 0.05$) with the IL-6 mRNA expression immediately after the acute exercise. Therefore, the higher muscle glycogen content was associated with lower IL-6 mRNA expression after exercise (Figure 1.31). In addition, the exercise-induced increase of plasma IL-6 was similar before and after training.

Interestingly, while the plasma IL-6 response to exercise seems to be reduced by training the muscular expression of the IL-6 receptor appears to be upregulated. After exercise training the basal IL-6 receptor α (IL-6rα) mRNA content in trained skeletal muscle is increased by ~100% (Akerstrom et al. 2009; Keller et al. 2005). It may, therefore, be possible that the downregulation of IL-6 is partially counteracted

by an increased expression of IL-6rα, and the sensitivity of IL-6 is increased (Figure 1.31).

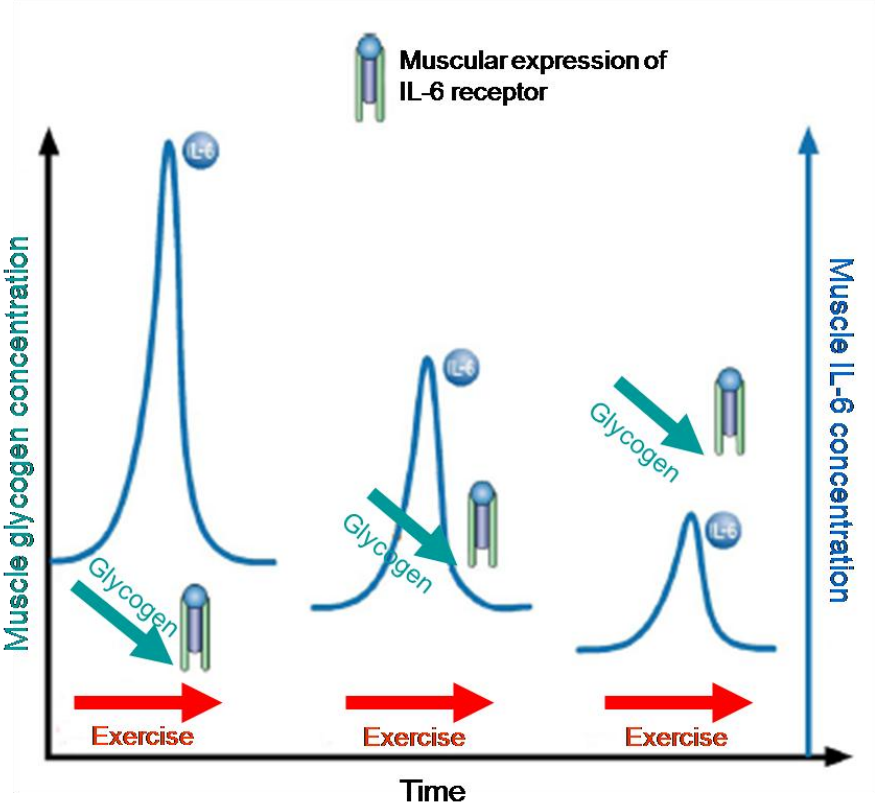


Figure 1.31 Schematic representation of how interleukin-6 (IL-6) may be regulated after exercise training. Exercise training results in an increase in glycogen synthase and the trained muscle will consequently store more muscle glycogen. During acute exercise the untrained muscle is highly dependent on glycogen as a substrate whereas training leads to increased β-oxidating enzymes and an enhanced capability to oxidise fat and, hence, to use fat as a substrate during exercise. The trained muscle, therefore, uses less glycogen during muscular contractions. The activation of muscle IL-6 is glycogen dependent. During prolonged and strenuous exercise there is a decrease in skeletal muscle glycogen content and IL-6 is upregulated at a faster rate. More IL-6 is produced at the same relative work compared with conditions with high muscle glycogen. During acute exercise this results in a lower plasma IL-6 concentration for trained compared to untrained muscles. The trained muscle may also be more sensitive to IL-6. Adapted from Pedersen and Febbraio (2008).

1.15.2 TRAINING AND OXIDATIVE STRESS

Several reports have now clearly shown that long term (weeks to months) endurance exercise training elevates key antioxidant enzymes in the diaphragms of rats compared to untrained controls (Figure 1.32; Powers et al. 1994b; Vincent et al.

1999; Vincent et al. 2000). These training-induced increases in diaphragmatic antioxidant capacity improve its ability to scavenge ROS and, thus, protect against exercise-induced oxidative stress (Powers and Shanely 2002). Interestingly, short-term endurance exercise (1 $h \cdot d^{-1}$ at ~70% $\dot{V}O_{2max}$ for 5 days) results in ~20% increases in manganese-superoxide dismutase activity which is similar in magnitude to that observed after 8 to 10 weeks of training (Vincent et al. 2000). In contrast, there are no significant increases in diaphragmatic antioxidant enzymes in dogs exposed to IRL (2 $h \cdot d^{-1}$ at 31−45% MIP for 2 weeks) compared to untrained controls (Barreiro et al. 2005). It could be postulated that this training load may not be a great enough stimulus for an adaptation. In support of this, those dogs that received the highest training loads also had the greatest amounts of oxidative stress evident.

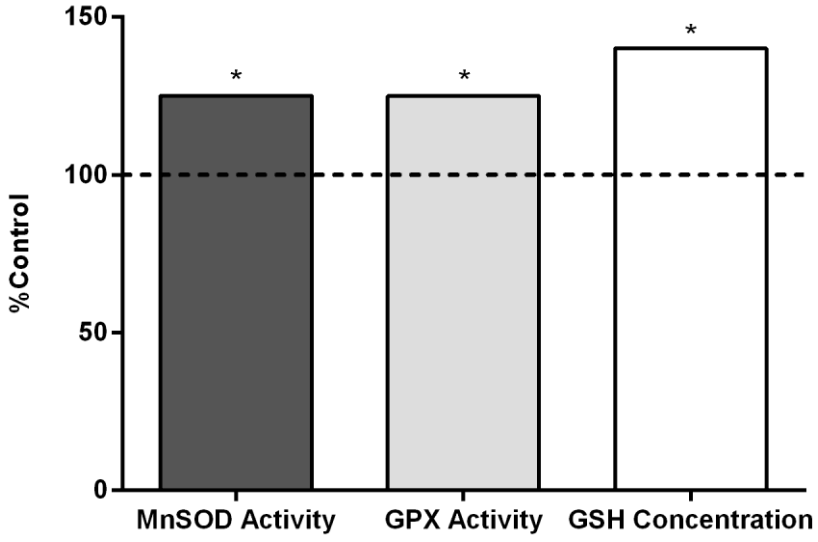


Figure 1.32 Costal diaphragm antioxidant enzyme capacity after endurance exercise training in rats. MnSOD, manganese superoxide dismutase; GPX, glutathione peroxidise; GSH, glutathione. Significant difference (from untrained control * $P < 0.05$). Values are mean from Powers et al. (1994b); Vincent et al. (1999); and Vincent et al. (2000). Adapted from Powers and Shanely (2002).

1.16 SYSTEMIC CYTOKINES AND OXIDATIVE STRESS IN AGEING

1.16.1 SYSTEMIC CYTOKINES IN AGEING

Aging is associated with an elevated systemic concentration of cytokines at rest. This has been termed systemic low-level inflammation and can be defined as a 2–4 fold increase in the resting concentration of inflammatory mediators (Bruunsgaard 2006). There is mounting evidence that suggests systemic low-level inflammation contributes significantly to the ageing process and age related diseases including, amongst others, atherosclerosis, cancer, dementia, metabolic syndrome, osteoporosis and sarcopenia (Chung et al. 2009). The mechanisms that underpin the role of systemic low-level inflammations in the development of age-related diseases are best understood by examining the metabolic effects of inflammation during acute illness. During acute illness, antigen-presenting cells recognise foreign elements and secrete pro-inflammatory cytokines IL-1β and TNF-α which assist the recruitment of T cells and the development of an antigen-specific response (Baumann and Gaulde 1994). In addition, these acute phase products can have a substantial effect on metabolism resulting in hyperglycaemia, insulin resistance and increased gluconeogenesis (Baumann and Gaulde 1994). An increase in these markers elevates bone reabsorbtion (Aris et al. 1998), dyslipidemia (Marik 2006), and proteolysis (Mitch and Goldberg 1996) whilst also increasing other parts of the inflammatory cascade that can have downstream biological effects. Whilst the changes during acute illness are much greater than those observed in ageing, it is suggested that long-term exposure to inflammation can contribute to the ageing process and age related diseases (Beavers et al. 2009).

Several studies have reported that compared to younger counterparts, older adults (65+ years) and the elderly (80+ years) have a higher resting systemic

concentration of cytokines such as TNF-α (Paolisso et al. 1998), IL-1β (Paganelli et al. 2002) and IL-6 (Bruunsgaard et al. 1999; Cohen et al. 1997; Ershler et al. 1993; Giuliani et al. 2001; Hager et al. 1994; Straub et al. 1998; Wei et al. 1992; Zietz et al. 2001). The resting plasma concentrations of TNF-α and IL-6 increase with age in randomly selected healthy humans (Figure 1.33; Bruunsgaard et al. 1999). However, some studies have failed to demonstrate age-related increases in TNF-α (Fagiolo et al. 1993; Peterson et al. 1994) and IL-6 (Beharka et al. 2001; Peterson et al. 1994). The differences in findings may relate to variations in the sensitivity of assays used, a lack of statistical power, differences in the age of study populations or differences in the health status of study participants (Bruunsgaard 2006). Indeed, the plasma IL-6 concentrations from randomly selected elderly participants are higher than very healthy elderly individuals selected in accordance with the SENIEUR (healthy ageing) protocol (10.3 ± 0.7 vs. 1.8 ± 0.3 $pg \cdot mL^{-1}$), demonstrating a strong influence of health status on resting systemic IL-6 concentrations (Baggio et al. 1998).

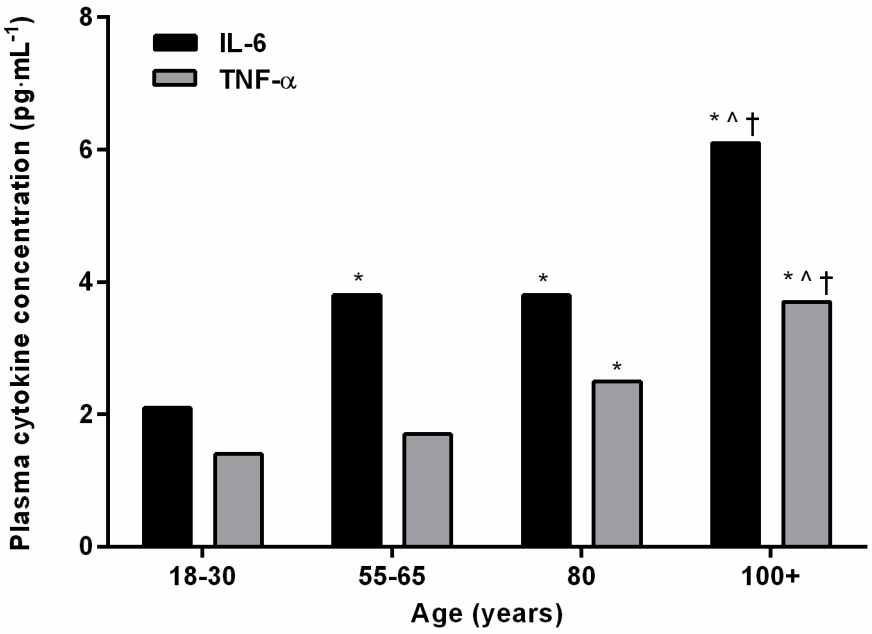


Figure 1.33 Plasma concentration of interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) at rest in healthy humans of increasing age. Significant difference ((P < 0.05) of 18–30 years from older age groups * 55–65 years; ^ 80 years; and † 100+ years). Values are mean. Adapted from Bruunsgaard et al. (1999).

1.16.2 BREATHING-INDUCED CYTOKINES IN AGEING

No studies have examined whether the respiratory muscles contribute to the systemic inflammation observed with ageing. In peripheral skeletal muscle, findings are equivocal to whether mRNA expression of IL-1β and TNF-α at rest increases with age (Hamada et al. 2005; Leger et al. 2008; Przybyla et al. 2006; Raue et al. 2007). Furthermore, IL-6 mRNA expression at rest is similar between younger and older males (Hamada et al. 2005; Pedersen et al. 2004; Przybyla et al. 2006; Trenerry et al. 2008). These findings suggest that in skeletal muscle IL-1β and TNF-α may, but IL-6 probably does not, contribute to the systemic inflammation observed with ageing.

Yende et al. (2006) have shown in 2005 healthy older adults (73 years) without obstructive lung disease that plasma IL-6 concentrations are not correlated with MIP. They were, however, inversely correlated with the percent of predicted

values of FEV_1. This finding suggests that plasma IL-6 concentrations are associated with age-related changes in lung function (but not inspiratory muscle strength) in healthy older adults.

1.16.3 OXIDATIVE STRESS IN AGEING

The mitochondrial theory of ageing hypothesises that ageing is related to an accumulation of ROS mediated oxidative damage and to a functional decline in the mitochondria (Wallace 2005). It is generally accepted that ROS increase with age and they play significant role in the ageing process (Fulle et al. 2004). This is especially true in skeletal muscle where there is a pronounced generation of free radicals due to the high amount of O_2 consumption compared to other tissues, and which results in higher levels of ROS (Fulle et al. 2004).

1.16.4 BREATHING-INDUCED OXIDATIVE STRESS IN AGEING

Indeed, levels of ROS (reactive carbonyls and malondialdehyde-protein adducts) and reactive nitrogen species (3-nitrotyrosine), and the activity of antioxidant enzymes (manganese-superoxide dismutase and catalase) are significantly higher in the external intercostals (taken with a biopsy) of older than younger adults (68 vs. 25 years) (Barreiro et al. 2006). Marzani et al. (2005) have supported some, but not all, of these findings. The activity of antioxidant enzyme manganese-superoxide dismutase was higher and the ratio of reduced to oxidised glutathione was lower in the rectus abdominis of older compared to younger (66−90 vs. 18−48 years) adults. However, there were no other differences in all other antioxidant enzymes including catalase. The differences between the studies may be explained by several reasons, including the type and work undertaken by the muscle

analysed (inspiratory vs. expiratory), the fibre type makeup of the muscle, or that Marzani et al. (2005) reported that both older and younger participants had rectus abdominis biopsy before surgery and the condition that required them to have surgery may have increased and/or changed levels of oxidative stress. These findings do provide evidence that the respiratory muscles contribute to increased levels of oxidative stress that is observed in the ageing process.

1.17 General summary

Increased respiratory muscle work is encountered during strenuous whole-body exercise, and at rest in older adults and those with pulmonary limitations such as chronic obstructive pulmonary disease. When sufficiently strenuous it can result in diaphragmatic fatigue, increased blood lactate concentrations, and an alteration in respiratory muscle recruitment patterns. Increased respiratory muscle work also elevates cytokines IL-6 and IL-1β within the respiratory muscles and systemically. There is mounting evidence that inflammation contributes significantly to the ageing process and age related diseases. Enhanced oxidative stress, glycogen depletion and diaphragmatic fatigue are all potential stimuli for this production. Whole-body exercise training can attenuate systemic inflammation and oxidative stress in younger adults during exercise and in older adults who experience this at rest. An attenuation of muscle glycogen or increases in antioxidant enzymes may explain such reductions. Inspiratory muscle training (IMT) may also elicit similar adaptations in the inspiratory muscles, and thus also attenuate these markers.

1.18 Research aims

In younger adults the research aims were to evaluate whether:

(I) The respiratory muscles contribute to exercise-induced increases in systemic cytokines and oxidative stress.

(II) An increase in systemic cytokines and oxidative stress is related to diaphragmatic fatigue.

(III) IMT attenuates systemic cytokines and/or oxidative stress during whole-body exercise or a volitional mimic at rest of exercise hyperpnoea.

(IV) IMT alters an estimation of the maximum lactate steady-state and respiratory muscle recruitment patterns during the lactate minimum test.

In older adults the research aims were to evaluate whether:

(V) IMT attenuates systemic cytokines and/or oxidative stress at rest.

CHAPTER 2 – GENERAL METHODS

2.0 YOUNGER PARTICIPANT PREPARATION

Younger participants provided written, informed consent to participate in the research which was approved by the Nottingham Trent University ethics committee. A self-reporting medical questionnaire confirmed that participants were non-smokers, free from illness and injury and were not taking any medication (including supplements or antioxidants) during the study (Appendix 1). Each participant completed a 24 h estimated diet record prior to their first trial which was then replicated prior to all subsequent trials. Throughout each study, participants were instructed to adhere to their habitual training regimen and not to engage in any strenuous exercise the day preceding and the day of a trial. Participants arrived at the laboratory 4 h postprandially having abstained from alcohol and caffeine in the 24 h before testing.

2.1 ANTHROPOMETRIC MEASUREMENTS

Body mass was measured to the nearest 0.1 kg using a calibrated electronic scale (Tanita Body Composition Analyser/Scale; Tanita UK Ltd, Yiewsley, UK) whilst participants were in lightweight clothing and barefoot (Eston 2008). Height was measured with a portable stadiometer (Seca Stadiometer; Seca Ltd, Birmingham, UK) while participants were looking straight ahead, standing barefoot, heels together, and with arms hanging naturally by their sides (Eston 2008).

2.2 LODE EXCALIBUR SPORT CYCLE ERGOMETER

During volitional hyperpnoea trials participants were seated on, or during exercise trials participants cycled on, an electromagnetically braked cycle ergometer (Excalibur Sport; Lode, Groningen, The Netherlands; Figure 2.1). The cycle

ergometer was externally calibrated (Lode). The ergometer was set in hyperbolic mode in which power output was constant and independent of pedal cadence. The handlebars and saddle height, and their respective horizontal displacements were adjusted for each individual and these configurations were recorded to the nearest mm and replicated for subsequent trials. The handlebars of the ergometer were modified with time trial bars (Aeroforce EA70 Clip on bar; Easton, CA, USA). The pedals on the cycle ergometer had toe clips and slots for cleated shoes. Participants could use either option, but once they had selected one were instructed to adhere to this option for the duration of the study. Pedal cadence was displayed at all times on a digital screen mounted on the handlebars. The power output of the cycle ergometer was controlled on an external personal computer running specific software on which exercise protocols were programmed to the nearest 1 W and 1 s (Lode ergometery manager, Version 5.18.20; Lode).

Figure 2.1 Experimental setup using cycling exercise or volitional hyperpnoea (Chapters, 3, 4 and 6), including electromagnetically braked cycle ergometer, breath by breath analyser (right hand computer), balloon pressure catheter system (left hand computer) and venous cannula (right hand).

2.3 PULMONARY FUNCTION: DYNAMIC SPIROMETRY

Pulmonary function was assessed according to published guidelines (Miller et al. 2005) using a pneumotachograph (Pneumotrac; Vitalograph, Buckingham, UK) calibrated with a 3 L syringe. Participants performed manoeuvres wearing a noseclip and standing upright. A minimum of 3 and a maximum of 8 flow-volume loops were performed to determine forced vital capacity (FVC), FEV_1, PEF and peak inspiratory flow (PIF) rates until within and between-manoeuvre criteria were satisfied, i.e., two largest recordings of FVC and FEV_1 within 100 mL of each other (Miller et al. 2005). A 10 s maximal voluntary ventilation (MVV_{10}) procedure was performed to determine the maximal breathing capacity. A minimum of 2 MVV_{10} procedures were

performed and continued until repeat measurements were within 20% of each other (Miller et al. 2005). The highest value recorded for the flow-volume loop and MVV_{10} were used for subsequent analysis (Miller et al. 2005). Spirometry data was compared to normal values provided by referenced equations. For younger participants, FVC, FEV_1, FEV_1/FVC and PEF were compared to Quanjer et al. (1993). For older participants, FVC, FEV_1 and FEV_1/FVC were compared to Falaschetti et al. (2004) and PIF and PEF to Garcia-Rio et al. (2004).

2.4 VOLITIONAL INSPIRATORY AND EXPIRATORY MUSCLE STRENGTH

A hand-held mouth pressure meter (MicroRPM; CareFusion, Hampshire, UK) measured MIP and MEP as an index of global inspiratory and expiratory muscle strength, respectively. The mouthpiece assembly incorporated a 1 mm orifice to prevent glottic closure during inspiratory efforts and minimise the contribution of the buccal muscles to inspiratory pressure development (Black and Hyatt 1969). Participants wore a noseclip and manoeuvres were performed whilst standing, sustained for at least 1 s and initiated from residual volume (MIP) or total lung capacity (MEP), respectively. Repeat efforts separated by 30 s were performed until three serial measures differed by no more than 10% or 10 cmH_2O, whichever was smallest (Brown et al. 2010; Johnson et al. 2012). The highest value recorded was used for subsequent analysis. Mouth pressure data was compared to normal values provided by referenced equations from Wilson et al. (1984) and Enright et al. (1995) for younger and older participants, respectively.

2.4.1 ACCURACY AND PRECISION OF THE MOUTH PRESSURE METER

The accuracy and precision of the mouth pressure meter was tested with a capacitive sensor with a pressure capacity of ≥ 1360 cmH_2O (1000 torr, Pirani strain gauge; MKS Barathon, MKS Instruments, MS, USA) and which was maintained at a constant temperature (55ºC) by a digital signal conditioner (Type 270B; MKS Instruments). A two-point calibration was performed on the strain gauge before commencement of each study. Firstly, the strain gauge pressure was set to zero, and secondly, to the atmospheric pressure provided by a laboratory based wall-based mercury manometer. The mouth pressure meter was connected via flexible tubing to the strain gauge and an electric syringe pump fitted with a 60 mL syringe (KDS 210C; KD Scientific, MA, USA) using a three way stopcock. The pump opened the syringe at a constant rate of 70.57 $mL \cdot min^{-1}$. Displacement of the syringe to multiple limits was repeated 10 times generating a range of pressures from 6 to 294 cmH_2O.

The relationship between the pressure measurements from the mouth pressure meter (P_{MOUTH}) and the calibrated strain gauge (P_{STRAIN}) is shown in Figure 2.1A. The correlation coefficient between P_{MOUTH} and P_{STRAIN} was excellent. The difference between P_{MOUTH} and P_{STRAIN} is plotted using a Bland and Altman plot (Bland and Altman 1986) in Figure 2.1B. Also shown in Figure 2.1B are the mean limits and the 95% confidence interval. This data demonstrates that the mouth pressure meter provides an accurate and precise measure of pressure given the narrow limits of agreement.

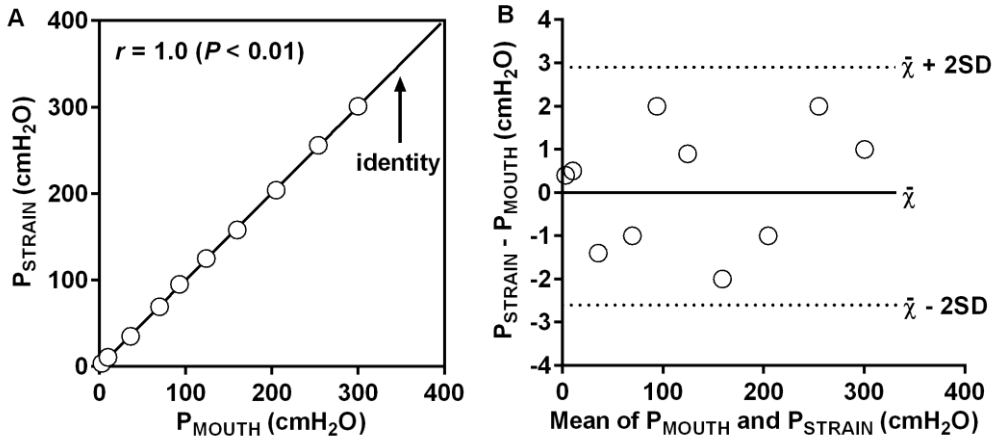


Figure 2.2 A) Strain gauge pressure (P_{STRAIN}) against mouth pressure meter pressure (P_{MOUTH}); B) difference in pressure between P_{STRAIN} and P_{MOUTH} against the mean of P_{STRAIN} and P_{MOUTH}. $\bar{\chi}$, mean.

2.5 ZAN 600 USB CPX BREATH BY BREATH ANALYSER

Pulmonary gas exchange and ventilatory responses were measured at the mouth using an online breath by breath gas analysis system operated by an integrated personal computer (ZAN 600USB; Nspire Health, Oberthulba, Germany; Figure 2.1). Pulmonary gases were continuously sampled and analysed using fast response selective analysers at a flow rate of 0.66 $L \cdot s^{-1}$ (O_2: amperometric solid state electrolyte sensor and CO_2: infrared spectroscopy). The expired gases were time aligned with expired airflow using specific data acquisition software (GPI version 3.0; Nspire Health) to provide breath by breath pulmonary gas exchange measurements expressed as standard temperature, pressure, dry (STPD). Expired volumes of air were measured using a pneumotachograph (Type II flow sensor; Nspire Health) with a low dead space (<40 mL) and expressed as body temperature, pressure, saturated (BTPS). According to the manufacturers guidelines the additional resistance provided by the flow pneumotachograph was 1.0, 1.7, 2.4 and 2.9 cmH_2O for flow rates of 6.2, 8.4, 10.0 and 11.2 $L \cdot s^{-1}$, respectively. The flow

pneumotachograph and gas analysers were calibrated before all trials using a 3 L syringe and gases of known concentrations (BOC Gases, Guildford, UK), respectively. The pneumotachograph was attached to a facemask (Vmask^{TM} model 7400; Hans Rudolph, KS, USA) with a low dead space (97 mL) and secured to the participants face with an elastic quick release mesh cap.

2.5.1 AGREEMENT BETWEEN ZAN 600 USB CPX AND A 'GOLD STANDARD'

The accuracy and precision of the online breath by breath expired gas analysis system was assessed against the closed circuit Douglas bag technique during exercise. The Douglas bag technique is the 'gold standard' for expired volumes and gas analysis (Bassett et al. 2001). Exercise was performed on an electromagnetically braked cycle ergometer (Excalibur Sport; Lode) with five constant power outputs (100, 130, 160, 190 and 220 W) each lasting 5 min. Expired air was collected in the final min of each power output. The breath by breath flow pneumotachograph was connected in series with a two-way non re-breathing valve (model 2730; Hans Rudolph) with the Douglas bag (non-permeable with a 200 L capacity) connected via corrugated tubing to the expiratory port. This permitted simultaneous measurement of expired air samples using both methods.

Douglas bag expired air samples were analysed for concentrations of O_2 and CO_2 using paramagnetic and infrared analysers, respectively (Series 1400; Servomex, Crowborough, UK), and calibrated immediately before analysis with gases of known concentrations (BOC Gases). Expired gas volumes were determined using a dry gas meter calibrated with a known volume of air (Harvard Apparatus, Kent, UK). $\dot{V}_E$, $\dot{V}O_2$ and carbon dioxide production ($\dot{V}CO_2$) were corrected to BTPS and STPD. The relationship between the pulmonary measurements from the ZAN

600 USB CPX and Douglas bag technique is shown in Figure 2.2. The correlation coefficient between the ZAN 600 USB CPX and Douglas bag technique was excellent for all the pulmonary measurements. The difference between ZAN 600 USB CPX and Douglas bag technique for pulmonary measurements is plotted using a Bland and Altman plot (Bland and Altman 1986) in Figure 2.3. Also shown in Figure 2.3 are the mean limits and the 95% confidence interval. This data demonstrates that the ZAN 600 USB CPX provides an accurate and precise measure of expired volumes and gas analysis given the narrow limits of agreement.

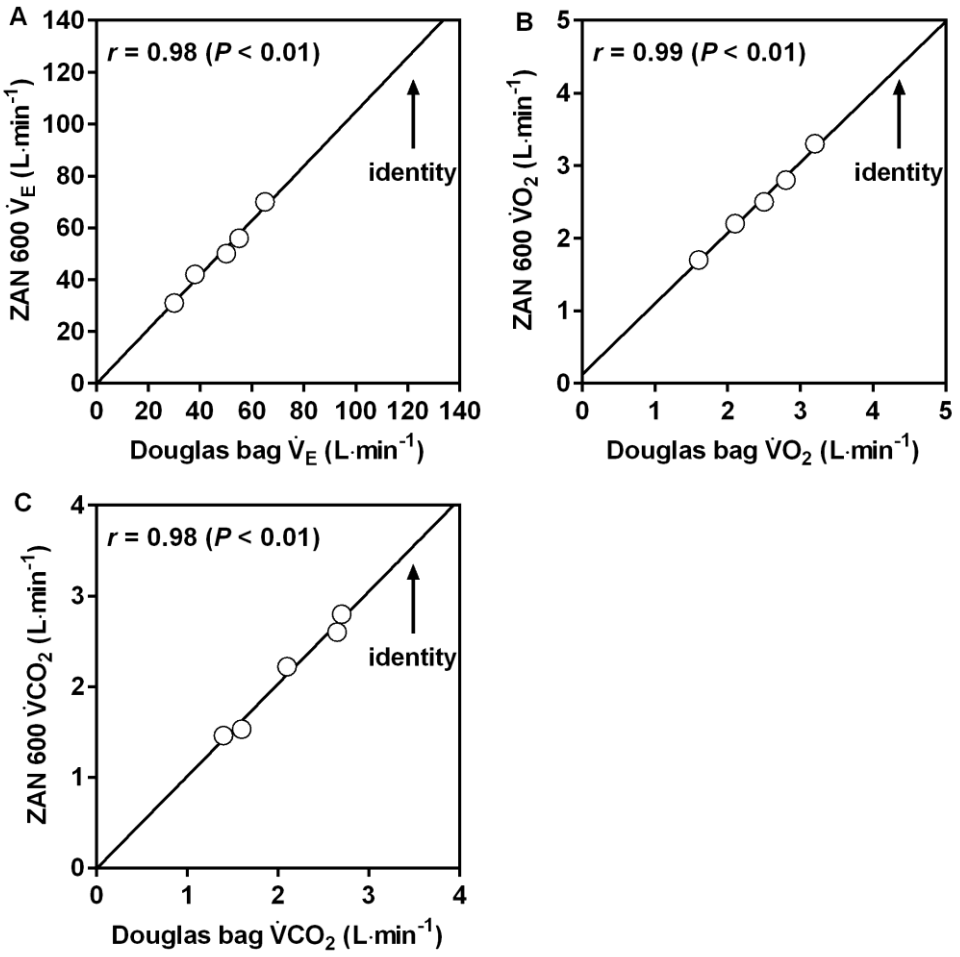


Figure 2.3 Relationship between ZAN 600 USB CPX and Douglas bag technique. A) Minute ventilation, $\dot{V}_E$; B) oxygen uptake, $\dot{V}O_2$; C) carbon dioxide production, $\dot{V}CO_2$.

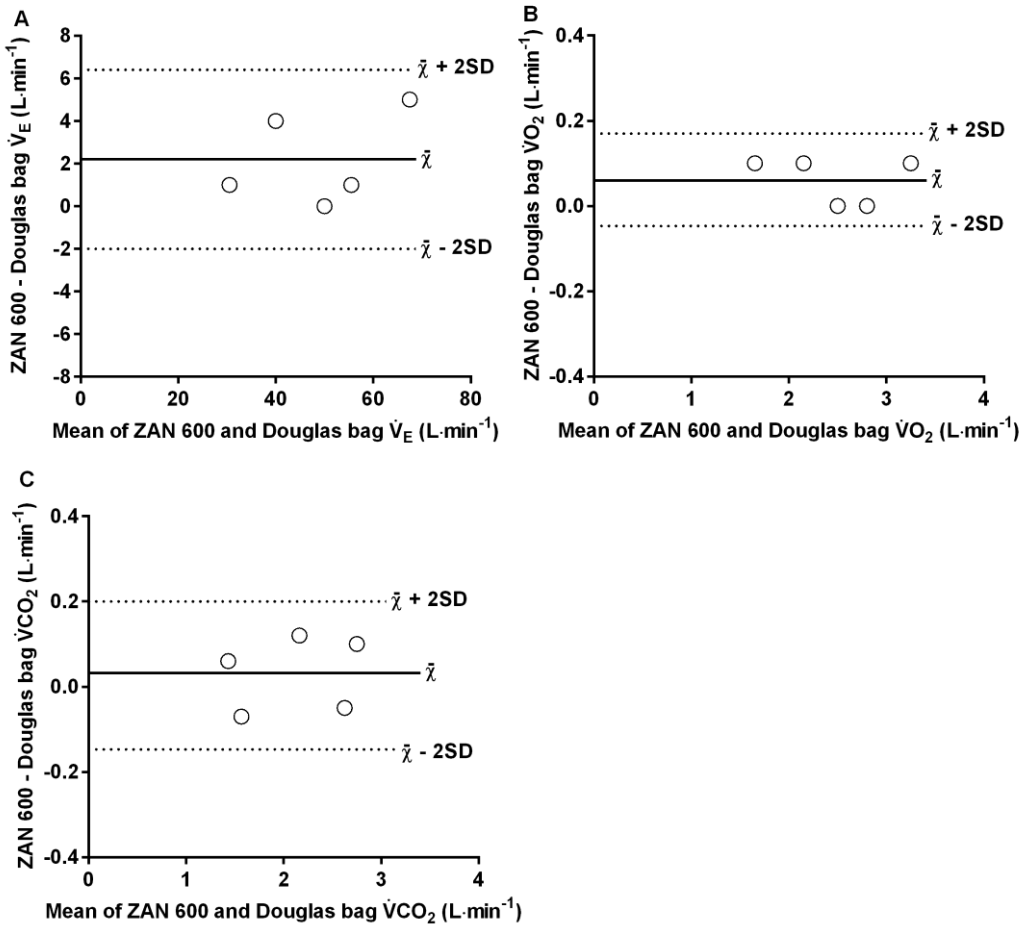


Figure 2.4 Difference in minute ventilation ($\dot{V}_E$; A), oxygen uptake ($\dot{V}O_2$; B) and carbon dioxide production ($\dot{V}CO_2$; C) against mean of ZAN 600 USB CPX and Douglas bag technique. $\bar{\chi}$, mean.

2.6 BALLOON PRESSURE CATHETER SYSTEM

P_e and P_g were measured using two thin walled (~0.6 mm) latex balloons sealed over a single polyethylene catheter (Nspire Health), which was attached to a differential pressure transducer (± 400 cmH_2O) (TSD104A; BIOPAC Systems, California, USA) and differential bridge amplifier (DA100C; BIOPAC Systems) (Figure 2.1). Pressure transducers were calibrated across the physiological range and were sampled at 200 Hz using an analogue-digital converter and recorded using specific data acquisition software (Acq*knowledge* version 3.7.3; BIOPAC systems). The balloon catheter was initially positioned in the stomach following local

anaesthesia of the nasal mucosa and posterior pharynx using 2% lidocaine (Instillagel®; Farco-Pharma GmbH, Cologne, Germany). The air within the oesophageal and gastric balloons was initially evacuated with a glass syringe and following a Valsalva manoeuvre, and were subsequently filled with 1 and 2 mL of air, respectively. The catheter was then withdrawn until, during repeated sniffs, P_e became negative. Subsequently, the catheter was withdrawn a further 10 cm. During the first experimental trial of the study the distance from the tip of the nares to the most distal point of the catheter was recorded and replicated in subsequent trials. P_{di} was calculated by subtracting P_e from P_g.

2.7 BILATERAL ANTERIOR MAGNETIC PHRENIC NERVE STIMULATION

BAMPS was applied using two double 25 mm coils connected to two Magstim 200[2] stimulators (Magstim Co, Dyfed, UK) that were triggered simultaneously. Participants initially rested for 20 min to minimise post-activation potentiation (Mador et al. 1994; Wragg et al. 1994). The location of each phrenic nerve was then identified by stimulating the area at the posterior border of the sternomastoid muscle at the level of the cricoid cartilage and determining the area associated with the largest P_{ditw}. This location was marked with indelible ink and used for subsequent stimulations. During stimulations, participants were seated upright with no abdominal binding (Koulouris et al. 1989; Man et al. 2002), wore a noseclip, and prior to stimulation were instructed to hold at functional residual capacity (Hubmayr et al. 1989; Smith and Bellemare 1987), which was inferred from visual feedback of P_e.

Skin surface EMG of the right costal diaphragm was recorded using bi-polar surface electrodes (self-adhesive, silver-silver chloride, 10 mm recording surface

diameter; Unilect, Worcestershire, UK) placed on the sixth and eighth intercostal spaces in the anterior axillary line (Glerant et al. 2006). Electrodes were positioned to optimise peak to peak amplitude of M-wave responses. EMG signals were passed through a differential amplifier (1902 Mk IV; Cambridge Electronic Design, Cambridge, UK), sampled at 2000 Hz using an analogue-digital converter (Micro 1401; Cambridge Electronic Design) and recorded using specific data acquisition software (Spike 2 version 5.12; Cambridge Electronic Design).

To determine supramaximal phrenic nerve stimulation, 3 single twitches were obtained every 30 s at intensities of 50, 60, 70, 80, 85, 90, 95 and 100% of maximal stimulator output. A plateau in M-wave and P_{ditw} responses with increasing stimulation intensities indicated maximum depolarisation of the phrenic nerves. The technical considerations regarding supramaximal stimulation have been described previously (Davies and White 1982; Taylor et al. 1996). P_{ditw} was assessed every 30 s using eight single stimulations at 100% of maximal stimulator output. To assess the potentiated P_{ditw} response, participants subsequently performed a 3 s maximal Müeller manoeuvre and ~5 s later a twitch was delivered. This procedure was repeated six times, with each measure separated by 30 s. A potentiated P_{ditw} has greater sensitivity to fatigue especially when the degree of fatigue is small (Laghi et al. 1995; Laghi et al. 1998). The average of the median three individual P_{ditw} responses was used for analysis. Diaphragm fatigue was defined as a >15% reduction in P_{ditw} compared to the baseline value (Kufel et al. 2002).

2.7.1 Reproducibility of bilateral anterior magnetic phrenic nerve stimulation

Four healthy males with normal respiratory function visited the laboratory on two separate visits and BAMPS was applied. Their age, body mass and height (mean

± SD) were 24 ± 3 years, 75 ± 7 kg and 179 ± 10 cm, respectively. Reproducibility was assessed using the coefficient of variation (CV). Planned pairwise comparisons were made with repeated measures *t*-tests with Bonferroni adjustment.

A plateau (i.e., no significant increase in amplitude with increasing stimulation intensity) in M-wave and P_{ditw} amplitude (Figure 2.4) in response to supramaximal BAMPS was observed for both visits indicating maximal depolarisation of the phrenic nerves. The plateau occurred at 85 to 90% of maximum stimulator output. The between-occasion reproducibility of the neuromuscular function measurements is shown in Table 2.1. There was excellent reproducibility for P_{ditw}, and to a lesser extent M-wave measurements. End-expiratory P_e and M-wave (potentiated) responses were less reproducible. End-expiratory P_e represents the pressure at an estimated functional residual capacity. Since this volume is estimated by sense alone, and without a target, there is variability in the measurement. A variability of 0.2−0.5 cmH_2O has been previously reported (Taylor and Romer 2009). The variability in potentiated M-wave responses is due to the Müeller manoeuvre which is effort dependent. To the author's knowledge there is no previous reproducibility measurements published on the M-wave responses after potentiated stimuli. The within-occasion between twitch CV for P_{ditw} and M-wave responses (mean of both visits) was 4.1% and 12.4% for non-potentiated and 4.4% and 12.2% for potentiated stimuli, respectively.

Table 2.1 Between-occasion reproducibility of neuromuscular function measurements.

	Visit 1	Visit 2	CV (%)
Non-potentiated			
End-expiratory P_e (cmH_2O)	-5.4 ± 2.3	-4.8 ± 2.5	21.9
End-expiratory P_g (cmH_2O)	7.5 ± 1.5	8.9 ± 1.7	12.7
P_{ditw} (cmH_2O)	15.7 ± 6.0	17.1 ± 5.4	5.67
M-wave (mV)	1.3 ± 0.9	1.5 ± 1.5	10.4
Potentiated			
End-expiratory P_e (cmH_2O)	-6.0 ± 1.6	-6.3 ± 2.4	17.2
End-expiratory P_g (cmH_2O)	7.0 ± 2.1	8.3 ± 1.7	12.7
P_{ditw} (cmH_2O)	25.0 ± 11.7	25.6 ± 9.6	5.21
M-wave (mV)	1.4 ± 0.7	1.8 ± 2.0	24.9
P_{dipeak} during Müller manoeuvre (cmH_2O)	118 ± 20	120 ± 22	2.80

CV, coefficient of variation; P_e, oesophageal pressure; P_g, gastric pressure; P_{ditw}, transdiaphragmatic twitch pressure; P_{dipeak}, peak transdiaphragmatic pressure.

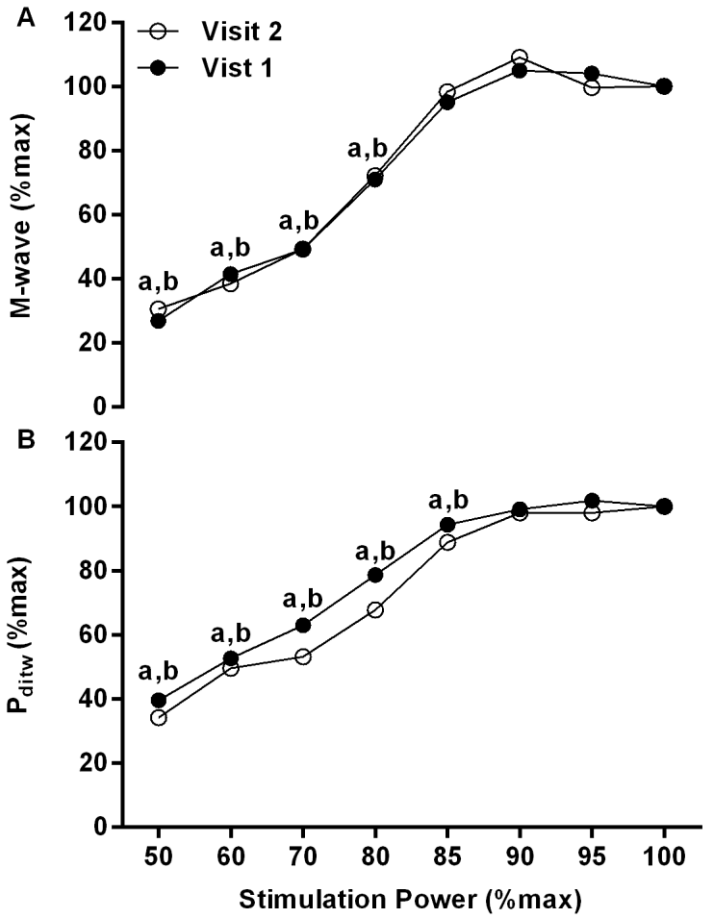


Figure 2.5 M-wave (A) and transdiaphragmatic twitch pressure (P_{ditw}; B) responses to supramaximal BAMPS. Significant difference (from values at 100% of stimulators power output ($P < 0.05$) for [a] visit 1 and [b] visit 2). Values are mean.

2.8 VOLITIONAL HYPERPNOEA

During volitional hyperpnoea participants mimicked breathing ($\dot{V}_E$, f_B, and T_I/T_{TOT}) and respiratory muscle recruitment (P_{dipeak}) patterns obtained during achieved during cycling exercise at the participants lactate minimum power (Chapter 3) and commensurate with heavy whole-body exercise (Chapter 4). An audio metronome paced f_R and T_I/T_{TOT}, and real-time visual feedback of $\dot{V}_E$ and P_{dipeak} was provided throughout. During volitional hyperpnoea trials, a two-way non-rebreathing valve (model 2730; Hans Rudolph) was attached distally to the pneumotachograph

and a 1.5 m length of wide-bore tubing was connected to the inspiratory port to allow CO_2 to be added into this tubing to increase the fraction of inspired carbon dioxide (F_ICO_2) and retain end-tidal and, consequently, PCO_2 at levels commensurate with rest (Brown et al. 2008; Johnson et al. 2006).

2.9 BLOOD SAMPLING FOR YOUNGER PARTICIPANTS

Arterialised venous blood was sampled from a dorsal hand vein via an indwelling 21-G teflon venous cannula (Surflo-W; Terumo, Leuven, Belgium; Figure 2.1). The cannula was fitted with a 3-way stopcock valve (Becton Dickenson UK Ltd, Oxford UK) and secured to the hand using adhesive medical tape. This method of arterialised venous blood sampling has been shown to provide excellent agreement with arterial blood for measures of $[La^-]_B$, PCO_2 and pH during both steady-state exercise and maximal incremental exercise until volitional tolerance (Forster et al. 1972; McLoughlin et al. 1992). Prior to cannulation, arterialisation was ensured by immersing the hand in water at a temperature of ~40°C for 10 min and during trials by warming the hand using a stand mounted infrared lamp (Infraphil HP3614; Phillps, Amsterdam, The Netherlands). Following cannulation, a 5 mL intravenous infusion of 0.9% sodium chloride (Mini-Plasco Saline; Braun, Melsungen, Germany) was performed to maintain patency. Immediately before all blood sampling, residual fluids were withdrawn from the cannula and stopcock using a 1 mL syringe. On completion of a trial, the cannula was removed and medical gauze (Topper 8; Johnson & Johnson Ltd, Skipton, UK) was applied under firm pressure to the puncture site for a minimum of 10 min to avoid superficial haematoma.

2.10 BLOOD LACTATE CONCENTRATION

1 mL arterialised venous blood samples were drawn into a syringe and were immediately transferred into sodium-heparinised 20 μL end-to-end capillary tubes (Biosen; EKF Diagnostics, Barleben, Germany). Capillary tubes were subsequently placed into a 1 mL micro test tube filled with lactate haemolysing solution (Biosen; EKF Diagnostics) and shaken vigorously for ~10 s. $[La^-]_B$ was determined using an automated analyser (Biosen C_line Sport; EKF Diagnostics). The lactate contained in the sample is converted enzymatically by the immobilised lactate oxidase on the chip sensor of the analyser. The bicarbonate generated by the reaction is detected at the electrode. The amperometric signal (sensor current) is proportional to the lactate concentration in the sample. The analyser was calibrated before use with a standard solution of a known concentration of lactate (12 $mmol \cdot L^{-1}$). The within sample CV for $[La^-]_B$ was 1.9% (n = 10 samples).

2.11 BLOOD GASES

1.5 mL arterialised venous blood samples were drawn into a syringe that contained sodium-heparin (PICO 50; Radiometer, Copenhagen, Denmark) and the syringe was inverted 8 times. The blood sample was subsequently aspirated into the injection port of an automated blood gas analyser (ABL 520; Radiometer) for the determination of PCO_2 and pH. The analyser aspirates the blood into a measuring chamber which has ion selective electrodes. The pH electrode compares a potential developed at the electrode tip with a reference potential and the resulting voltage is proportional to the concentration of hydrogen ions. The PCO_2 electrode is a pH electrode with a silicone rubber CO_2 semi-permeable membrane covering the tip. CO_2 combines with water in the space between the membrane and the electrode tip to

produce free hydrogen ions in proportion to the PCO_2. The blood gas analyser performed automated calibrations at 2 h intervals. Additionally, four levels of quality control solutions (QUALICHECK 3+; Radiometer) were introduced monthly into the analyser to ensure accuracy and precision. The within sample CV was 2.2 and 3.4% for PCO_2 and pH, respectively (n = 10 samples).

2.12 ENZYME LINKED IMMUNOSORBANT ASSAY

5 mL blood samples were drawn into a syringe and immediately transferred into pre-cooled tubes containing 1.6 $mg \cdot mL^{-1}$ of K_3E ethylenediaminetetraacetic acid (EDTA) (SARSTEDT, Leicester, UK). Tubes were gently inverted several times and then centrifuged for 15 min at 1000 x g and 5ºC. The plasma supernatant was subsequently removed and stored at -80ºC until further analysis. Plasma cytokines in Chapters 3 and 4 were analysed using a commercial solid phase sandwich ELISA (Figure 1.18) (Quantikine; R&D Systems, Abingdon, UK). Plasma cytokine concentrations were read in duplicate using an automated absorbance microplate reader (ELx800; BioTek, Vermont, USA).

2.13 MEASUREMENT OF SYSTEMIC OXIDATIVE STRESS: COMET ASSAY

The measurement of systemic oxidative stress was undertaken using the comet assay which can determine levels of DNA damage at the level of the individual eukaryotic cell. In its basic form, the comet assay measures DNA strand breaks and alkali-labile sites, i.e. apurinic/apyrimidinic sites, or baseless sugars. These cannot be specifically related to oxidation, since they result from various forms of damage, and might also represent intermediates in the repair process.

The comet assay has been extensively utilised at Nottingham Trent University (see Marthandan et al. 2011 and 2013 for most recent publications) and when the same investigator undertakes scoring of slides the inter-assay CV is <10% (Moller 2006). Previous reports have shown that prolonged and strenuous whole-body exercise can cause oxidative DNA damage in PBMC which can be measured with the comet assay (Collins et al. 1993; Wagner et al. 2011). Therefore, the technique would be suitable to detect changes in respiratory muscle ROS production. The technique does, however, exhibit large inter-individual differences in the amount of DNA damage due to, amongst others, endogenous factors such as diet, PBMC composition, or exposure to exogenous and endogenous genotoxins, and to a lesser extent variations in the handling and scoring of PBMC which cannot be excluded from the technique (Moller 2006).

2.13.1 ISOLATION OF PERIPHERAL BLOOD MONONUCLEAR CELLS

PBMC were used for the comet assay and were isolated using density gradient centrifugation. 5 mL of heparinised venous blood and 5 mL of phosphate buffered saline were layered onto a lymphocyte separation medium (Ficoll-Paque Plus; Griener Bio-One, Stonehouse, UK) inside a Leucosep tube (Griener Bio-One, Stonehouse, UK) and centrifuged for 15 min at 800 x *g* and room temperature. The opaque mononuclear cell layer was aspirated and washed 3 times in phosphate buffered saline and centrifuged for 10 min at 200 x *g* and room temperature. The cells were cryopreserved in liquid nitrogen for subsequent analysis in a medium consisting of 10% Dimethyl sulfoxide (Sigma-Aldrich, Gillingham, UK), 20% foetal bovine serum (Invitrogen, Paisley, UK) and 70% X-Vivo 10 media (Lonza, Wokingham, UK).

2.13.2 COMET ASSAY

Levels of DNA damage (DNA single strand breaks and alkali labile lesions) in PBMC were determined using the alkaline comet assay (Singh et al. 1988) and the modified alkaline comet assay (Collins et al. 1993). In the modified comet assay, PBMC embedded on slides were treated with either formamidopyrimidineglycosylase (FPG) which recognises oxidatively modified purines (Boiteux et al. 1992), or with endonuclease III (ENDO III) which recognises oxidatively modified pyrimidines (Asahara et al. 1989). These enzymes nick DNA at the sites of oxidatively damaged nucleotides, creating single-strand breaks which can be detected with the alkaline comet assay. PBMC treated with 150 μM hydrogen peroxide for 5 min at 4ºC (to induce oxidative damage) were used as internal positive controls in the modified alkaline comet assay.

The comet assays were performed at 4ºC to minimise the repair of existing basal levels of DNA damage present in PBMC. PBMC (2×10^4 cells·gel^{-1}) were embedded in a 1% agarose gel on frosted microscope slides, and lysed for 1 h in a high salt alkaline buffer (2.5 M sodium chloride, 0.1 M EDTA, 0.01 M Tris, 1% (v/v), Triton X-100, pH 10). For the modified comet assays, slides were equilibrated in enzyme buffer (0.04 M HEPES, 0.1 M potassium chloride, 0.5mM EDTA, 0.2 mg·mL^{-1} bovine serum albumin, pH 8.0) prior to the application of FPG or ENDO III. Slides treated with the lesion specific enzymes were incubated at 37ºC in a humid dark chamber for 45 min. Following enzyme treatment (or immediately after alkaline lysis for the alkaline comet assay) the slides were placed in electrophoresis buffer (0.3 M sodium hydroxide, 1 mM EDTA, pH 13) for 20 min to allow alkaline unwinding of the DNA, and then electrophoresed at 25 V and 300 mA for 30 min. Subsequently, slides were neutralised (0.4 M Tris, pH 7.5) and stained (50 μg·mL^{-1}

ethidium bromide) to visualise DNA. Stained slides were digitally analysed using ultraviolet microscopy (Carl Zeiss Ltd, Welwyn Garden City, UK) and scored (50 PBMC per slide) using analysis software (Komet 5.5; Andor Bio Imaging, Nottingham, UK) (Figure 2.5).

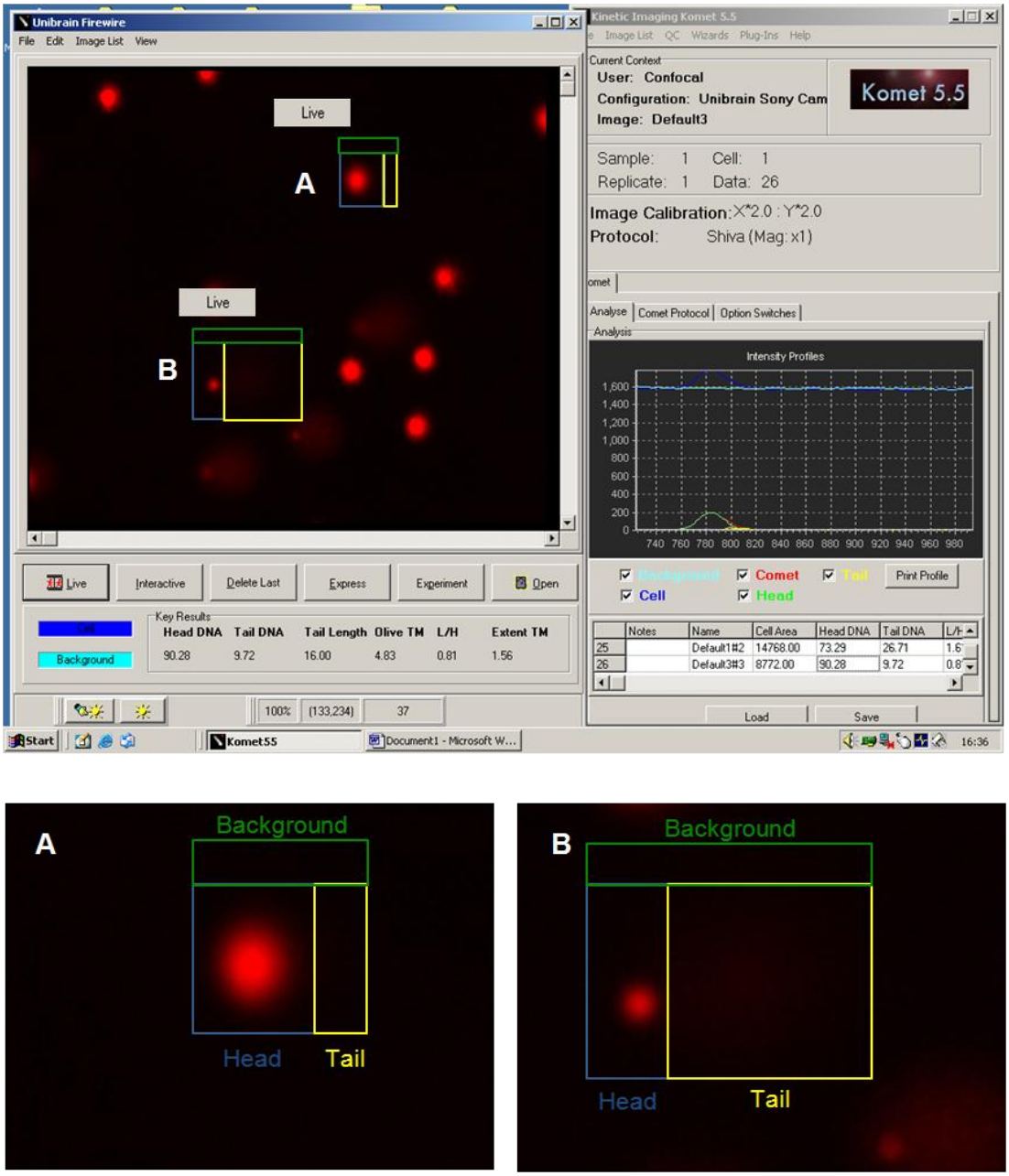


Figure 2.6 Comet analysis. The damaged DNA inside the peripheral blood mononuclear cell (PBMC) nucleus is pulled from anode to cathode during electrophoresis. The slides are subsequently stained to visualise the DNA and they are viewed using ultraviolet microscopy. The top panel shows the analysis software where a live image is visualised and two separate PBMC have been randomly chosen and scored. Three separate boxes are placed around the PBMC to measure the intensity of light emitted from the background (green), the comet head - intact DNA (blue), and the comet tail - damaged DNA (yellow). The software calculates the light emitted from the head and tail as a percentage and subtracts the light emitted from the background. The bottom panel shows A) 90% DNA of the the head of the comet and 10% in the tail - 10% DNA damage; and B) 74% DNA of the the head of the comet and 26% in the tail - 26% DNA damage.

2.14 RATING OF PERCEIVED EXERTION

RPE for leg discomfort and dyspnoea was measured using Borg's modified CR10 scale (Borg 1982). For trials involving younger participants, both scales were mounted onto a magnetic board that faced the participant throughout the trial and measurements were taken via the participant moving a magnetic counter to the desired integer. For trials involving older participants, a single scale was used and participants pointed to the correct integer. Both sets of participants were familiarised with scales and were encouraged to provide a differential response relative to leg discomfort and dyspnoea.

2.15 INSPIRATORY MUSCLE TRAINING

IMT participants performed 30 consecutive dynamic inspiratory efforts twice daily for 6 weeks (younger) or 8 weeks (older) using an inspiratory pressure-threshold device (POWERbreathe® Classic series 1st generation; Gaiam, UK). The initial training load was 50% MIP. Thereafter, participants periodically increased the load so that 30 manoeuvres could only just be completed. Each inspiratory effort was initiated from residual volume and participants strove to maximise V_T. This regimen is known to be effective in eliciting an adaptive response (Brown et al. 2008; Brown et al. 2010; Brown et al. 2012; Romer and McConnell 2003).

2.16 LACTATE MINIMUM TEST

The lactate minimum test was performed following the procedures of Johnson et al. (2009) who reported excellent agreement between lactate minimum and MLSS powers (mean bias ± 95% limits of agreement: 2 ± 22 W). The test comprised three consecutive phases: (i) lactate elevation phase comprising maximal

incremental exercise; (ii) 8 min recovery phase at 60 W; and (iii) incremental phase comprising five consecutive 4 min stages at intensities of 45, 50, 55, 60, and 65% of the $\dot{W}_{max}$ achieved during the lactate elevation phase. During the lactate elevation phase power output was increased by 10 W every 15 s so that exercise intolerance occurred in approximately 10 min. The highest oxygen uptake recorded in any 30 s period defined $\dot{V}O_{2peak}$. During the incremental phase, arterialised venous blood samples were taken in the final seconds of each stage, and the lactate minimum power (i.e. estimated MLSS power) was calculated as the nadir of a 2nd order polynomial fitting the $[La^-]_B$ against power data.

CHAPTER 3 – INSPIRATORY MUSCLE TRAINING REDUCES PLASMA INTERLEUKIN-6 CONCENTRATION DURING CYCLING EXERCISE BUT NOT A VOLITIONAL MIMIC OF THE EXERCISE HYPERPNOEA

3.0 INTRODUCTION

The plasma concentration of several cytokines increases following prolonged and strenuous whole-body exercise, with the myokine IL-6 demonstrating the largest increase (Pedersen and Febbraio 2008). Contracting skeletal muscle accounts for most (but not all) of the exercise-induced increase in plasma IL-6 (Steensberg et al. 2000), which acts to stimulate lipolysis (Wolsk et al. 2010), hepatic glucose output (Febbraio et al. 2004), and glucose uptake in the contracting myocytes (Carey et al. 2006).

Given their relatively small mass (~960 g; Freedman et al. 1983) it may seem unlikely that the respiratory muscles contribute to exercise-induced increases in plasma IL-6. However, Vassilakopoulos et al. (1999) reported a 120% increase in plasma IL-6 concentration in young and healthy humans following IRL at 75% MIP. The increase in plasma IL-6 concentration was attributed to increased rates of IL-6 release from the inspiratory muscles, which is supported by the observation of increased IL-6 mRNA and protein expression in the diaphragm of rats exposed to IRL (Sigala et al. 2011; Sigala et al. 2012; Vassilakopoulos et al. 2004). Although these findings have clinical relevance to those with asthma and obstructive lung disease, they cannot be generalised to healthy humans performing whole-body exercise as, compared to IRL, spontaneous exercise hyperpnoea elicits different breathing and respiratory muscle recruitment patterns (Clanton et al. 1985b; Clanton et al. 1990; Eastwood et al. 1994).

Vassilakopoulos et al. (1999 and 2002) also suggested that the increase in plasma IL-6 concentration (and other cytokines) may be due to inspiratory / diaphragm muscle fibre injury / fatigue since IRL was performed until task failure, which occurred after 54 ± 10 min. However, inspiratory muscle fatigue was assessed

using a volitional measure of MIP, which may decline following IRL until task failure due to a decrease in central drive globally rather than diaphragm fatigue (ATS/ERS 2002; McKenzie et al. 1997). The question of whether respiratory muscles release IL-6 during exercise, and if this is related to diaphragm fatigue, may be better addressed using volitional hyperpnoea at rest. In this model validity is improved by mimicking the breathing and respiratory muscle recruitment patterns experienced during whole-body exercise (Babcock et al. 1995; Klas and Dempsey 1989) and the presence of diaphragm fatigue can be measured more objectively by using bilateral phrenic nerve stimulation (ATS/ERS 2002).

Recent evidence suggests that the plasma IL-6 response to exercise is attenuated following resistance (Izquierdo et al. 2009) and endurance (Akerstrom et al. 2009; Croft et al. 2009; Yfanti et al. 2012) training. Increases in basal levels of IL-1rα (Akerstrom et al. 2009; Keller et al. 2005) or an increase in antioxidant enzymes (Yfanti et al. 2012) may explain such reductions. Alternatively, a strong stimulus for IL-6 production by skeletal muscle fibres is muscle glycogen depletion (Keller et al. 2005; Steensberg et al. 2001), which is attenuated in trained muscle (Croft et al. 2009; Fischer et al. 2004b) secondary to training-induced increases in mitochondrial density (Hoppeler and Fluck 2003). IMT may elicit similar morphological adaptations in the inspiratory muscles (Brown et al. 2008; Brown et al. 2010; Brown et al. 2012; Ramirez-Sarmiento et al. 2002), and thus also attenuate the plasma IL-6 response to whole-body exercise and/or volitional hyperpnoea.

Therefore, the aim of this study was to examine whether the respiratory muscles contribute to exercise-induced increases in plasma IL-6 concentration, if this is related to diaphragm fatigue, and whether IMT attenuates the plasma IL-6 response to whole-body exercise and/or volitional hyperpnoea.

3.1 METHODS

3.1.1 PARTICIPANTS

Twelve non-smoking recreationally active males provided written, informed consent to participate in the study, which was approved by the Nottingham Trent University ethics committee (Appendix 1 and 2). Participants followed the pre-experimental instructions outlined in Section 2.0.

3.1.2 EXPERIMENTAL DESIGN

Participants attended the laboratory on 5 separate occasions, before and after a 6 week intervention. Each laboratory visit was separated by 48 h and took place at the same time of day. During the first visit participants were familiarised with all testing procedures and pulmonary function and MIP were measured. During the second visit participants performed a cycling lactate minimum test to estimate MLSS power. Subsequent experimental trials lasted 1 h and comprised: (i) passive rest (PASSIVE); (ii) cycling exercise at the participants lactate minimum power (EX); and (iii) volitional hyperpnoea at rest whereby participants mimicked the breathing and respiratory muscle recruitment patterns achieved during EX (HYPEX). Since EX had to precede HYPEX, the experimental trials were only partially randomised. Following the final trial participants were randomly, and equally, divided into an IMT or placebo (PLA) group and completed a 6 week intervention. At least 48 h post-intervention, participants repeated the experimental trials in the same order as pre-intervention. All trials were performed on an electromagnetically braked cycle ergometer (Excalibur Sport; Lode).

3.1.3 PULMONARY FUNCTION AND MAXIMAL INSPIRATORY MOUTH PRESSURE

Pulmonary function and MIP were assessed according to the procedures stated in Sections 2.3 and 2.4, respectively.

3.1.4 LACTATE MINIMUM TEST

The lactate minimum test was performed following the procedures of Johnson et al. (2009) as stated in Section 2.16.

3.1.5 EXPERIMENTAL TRIALS

During experimental trials the configuration of the cycle ergometer and the body position adopted by each participant were identical to those adopted during the lactate minimum test. Each trial was preceded by a 5 min rest period and during PASSIVE participants remained seated on the cycle ergometer for the duration of the trial. During pre- and post-intervention EX trials participants cycled at their pre-intervention lactate minimum power and each participant adopted the same cycling cadence during all trials. During HYPEX participants mimicked the breathing ($\dot{V}_E$, f_B and T_I/T_{TOT}) and respiratory muscle recruitment (P_{dipeak}) patterns attained during pre-intervention EX. These variables were ensemble averaged into six 10 min blocks from the EX data and targets were updated during the HYPEX trial. During the post-intervention HYPEX trial participants mimicked the breathing and respiratory muscle recruitment patterns performed during the pre-intervention HYPEX trial. An audio metronome paced f_B and T_I/T_{TOT}, and real-time visual feedback of $\dot{V}_E$ and P_{dipeak} was provided throughout. All experimental trials were performed in an environmental chamber (Design Environmental WIR52-20HS; Design

Environmental, Gwent, UK) at 20ºC and 90% relative humidity to minimise mucosal drying of the airways during HYPEX.

3.1.6 FLOW, PULMONARY GAS EXCHANGE AND PRESSURE MEASUREMENTS

During all trials participants wore a facemask (model 7940; Hans Rudolph) connected to a pneumotachograph and ventilatory and pulmonary gas exchange responses were measured breath by breath (ZAN 600USB; Nspire Health). During experimental trials a two-way non re-breathing valve (model 2730; Hans Rudolph) was attached distally to the pneumotachograph and a 1.5 m length of wide-bore tubing was connected to the inspiratory port. During HYPEX CO_2 was added into this tubing to increase F_ICO_2 and retain end-tidal and, consequently, blood PCO_2 at levels commensurate with rest (Brown et al. 2008; Johnson et al. 2006). On the expiratory port of the two-way valve a Fleisch no. 3 pneumotachograph was attached and connected to a differential pressure transducer (± 2.5 cmH_2O) (TSD160A; BIOPAC Systems) and differential bridge amplifier (DA100C; BIOPAC Systems) to allow alignment of flow and pressure signals. P_e and P_g were measured according to the procedures stated in Section 2.6. P_{di} was calculated by subtracting P_e from P_g. P_{di} and P_e were integrated over the period of inspiratory flow and multiplied by f_B and labelled the diaphragm pressure-time product (PTP_{di}) and the inspiratory muscle pressure-time product (PTP_e), respectively. Non-physiological flows and pressures that resulted from swallowing, coughing and breath holding, were identified by visual inspection and removed. Breathing mechanics data were ensemble averaged into six 10 min blocks and used for subsequent analysis.

3.1.7 BILATERAL ANTERIOR MAGNETIC PHRENIC NERVE STIMULATION

BAMPS was applied according to the procedures stated in Section 2.7. Non-potentiated P_{ditw} and potentiated P_{ditw} was measured at baseline and within 15 (<15 min), 35 (<35 min) and 60 (<60 min) min after each experimental trial. Diaphragm fatigue was defined as a >15% reduction in P_{ditw} compared to the baseline value (Kufel et al. 2002).

3.1.8 BLOOD ANALYSES AND ADDITIONAL MEASUREMENTS

Arterialised venous blood was sampled from a dorsal hand vein via an indwelling 21-G cannula (McLoughlin et al. 1992). During the experimental trials blood samples were taken at rest, every 3 min for the initial 15 min, and every 5 min thereafter, and analysed immediately for PCO_2 and pH (ABL520; Radiometer), which were corrected for changes in rectal temperature (Squirrel 2020; Grant Instruments, Cambridge, UK). At rest and every 10 min during each trial $[La^-]_B$ was determined using an automated analyser (Biosen C_line Sport; EKF Diagnostics) and measurements were taken for f_C using short range telemetry (Polar S610; Polar, Kempele, Finland), estimated arterial oxygen saturation (SpO_2) using infrared fingertip pulse oximetry (Model 8600; Nonin, Minnesota, USA), and RPE for leg discomfort and dyspnoea using Borg's modified CR10 scale (Borg 1982). At rest, immediately after (0 h) and 1 (+1 h), 2 (+2 h), and 24 h (+24 h) after each experimental trial, 5 mL blood samples were taken for the measurement of plasma IL-6 concentration. Participants were instructed to not undertake any physical activity between +2 h and + 24 h. Blood was transferred into pre-cooled tubes containing 1.6 $mg \cdot mL^{-1}$ of K_3E EDTA (SARSTEDT) and immediately centrifuged for 15 min at 1000 x *g* and 5ºC. The plasma supernatant was subsequently removed

and stored at -80ºC until further analysis. Plasma IL-6 concentrations were measured in duplicate using a commercial solid phase sandwich ELISA (Quantikine HS; R&D Systems, Abingdon, UK). The assay has a detection limit of 0.039 $pg \cdot mL^{-1}$. To minimise the effect of inter-assay variation, pre- and post-intervention samples from both groups were measured during the same assay. The inter- and intra-assay CV for plasma IL-6 concentration was <10%.

3.1.9 INSPIRATORY MUSCLE TRAINING AND PLACEBO INTERVENTIONS

The IMT group performed training sessions according to the procedures stated in Section 2.15. The PLA group inhaled one puff twice daily for 6 weeks from a sham metered dose inhaler (Vitalograph). The inhaler contained compressed air only but was promoted to the participants as delivering a novel drug that reduces breathing-induced inflammation. During the post-intervention period the IMT and PLA groups performed their intervention 2 $d \cdot wk^{-1}$, which is sufficient to maintain improvements in inspiratory muscle function after IMT (Romer and McConnell 2003). These maintenance sessions were performed 48 h before and 48 h after experimental trials. All participants completed a training diary throughout the study to record adherence to the prescribed intervention and whole-body training sessions.

3.1.10 STATISTICAL ANALYSES

Statistical analyses were performed using SPSS for Windows (IBM, Chicago, Illinois, USA). Groups were compared for pre-intervention characteristics using independent *t*-tests. A two-way repeated measures analysis of variance (ANOVA) was used to test between-group effects due to 'treatment' (IMT vs. PLA) and within-group effects due to 'intervention' (pre- vs. post-'treatment'), 'trial' (EX vs.

HYPEX) or 'time' (10, 20, 30, 40, 50 and 60 min for physiological variables; 0 h, +1 h , +2 h and +24 h for plasma IL-6 data or baseline, <15 min, <35 min and <60 min for P_{ditw} data). Planned pairwise comparisons were made with repeated measures *t*-tests and with Bonferroni adjustment. Plasma IL-6 and $[La^-]_B$ responses were also analysed using a three-way repeated measures ANOVA ('treatment' x 'intervention' x 'time'). Reliability was assessed using a CV calculated from a pooled mean of both groups and all trials. Statistical significance was set at $P < 0.05$. Results are presented as mean ± SD.

3.2 RESULTS

Age (IMT vs. PLA: 27 ± 7 vs. 22 ± 3 yr), body mass (80 ± 6 vs. 74 ± 7 kg), height (181 ± 5 vs. 177 ± 4 cm) and $\dot{V}O_{2peak}$ (52 ± 6.2 vs. 47 ± 5.7 $mL \cdot kg^{-1} \cdot min^{-1}$) were not different between groups. Except for lactate minimum power, which was higher ($P < 0.01$) in the IMT group (199 ± 8 W) compared to the PLA group (170 ± 19 W), all pre-intervention measurements were not different between groups. In both groups all responses remained unchanged from rest during PASSIVE trials (data not shown).

3.2.1 PULMONARY FUNCTION AND MAXIMAL INSPIRATORY PRESSURE

Pre-intervention pulmonary function and MIP were within normal limits (Table 3.1). Compliance with the intervention was excellent in both IMT and PLA groups with 96 ± 1 and 93 ± 1% of sessions completed, respectively. Pulmonary function was unchanged in both groups post-intervention, whereas MIP increased by 24 ± 16% following IMT ($P < 0.01$). Inspection of training diaries revealed habitual whole-body training remained constant in both groups.

Table 3.1 Pulmonary function and maximal inspiratory pressure for IMT and PLA groups. Predicted values from Quanjer et al. (1993) and Wilson et al. (1984). Values are mean ± SD.

	IMT		PLA	
	Pre-intervention	Post-intervention	Pre-intervention	Post-intervention
FVC (L)	5.58 ± 0.44	5.65 ± 0.47	5.61 ± 0.67	5.49 ± 0.62
FVC (% Predicted)	104 ± 7	105 ± 5	105 ± 9	103 ± 8
FEV_1 (L)	4.49 ± 0.46	4.43 ± 0.20	4.53 ± 0.51	4.34 ± 0.57
FEV_1 (% Predicted)	100 ± 8	102 ± 7	100 ± 10	96 ± 12
FEV_1/FVC (%)	81 ± 6	81 ± 4	81 ± 6	80 ± 8
FEV_1/FVC (% Predicted)	99 ± 6	99 ± 5	98 ± 7	96 ± 9
MVV_{10} ($L \cdot min^{-1}$)	208 ± 2	206 ± 20	192 ± 33	196 ± 25
MVV_{10} (% Predicted)	111 ± 14	111 ± 11	102 ± 19	104 ± 14
MIP (cmH_2O)	168 ± 49	202 ± 39*	136 ± 32	139 ± 31
MIP (% Predicted)	157 ± 9	178 ± 34*	113 ± 26	115 ± 25

FVC, forced vital capacity; FEV_1, forced expiratory volume in 1 s; MVV_{10}, maximum voluntary ventilation in 10 s; MIP, maximal inspiratory pressure. Significant difference (from pre-intervention $^*P < 0.01$).

3.2.2 BREATHING AND RESPIRATORY MUSCLE RECRUITMENT PATTERNS

With the exception of PTP_{di}, which was higher during HYPEX compared to EX in IMT (main effect for trial, $P < 0.05$) and PLA (trial x time interaction, $P < 0.01$) groups, the pre-intervention exercise hyperpnoea was successfully mimicked during HYPEX (Table 3.2 and Figure 3.1). In the IMT group, the higher PTP_{di} during HYPEX compared to EX resulted in a higher PTP_{di}/PTP_e (main effect for trial, $P < 0.05$). Breathing and respiratory muscle recruitment patterns during EX and HYPEX were unchanged following both IMT and PLA. A representative breathing and respiratory muscle recruitment pattern trace is shown in Figure 3.2. The inter-trial CV for P_{dipeak} at rest was <7%.

Table 3.2 Physiological and perceptual responses to EX and HYPEX for IMT and PLA groups. Values are mean ± SD.

	EX		HYPEX	
	Pre-intervention	Post-intervention	Pre-intervention	Post-intervention
IMT				
$\dot{V}_E$ ($L \cdot min^{-1}$)	81.0 ± 13.0	75.1 ± 6.1	77.3 ± 15.1	77.0 ± 15.9
V_T (L)	2.41 ± 0.55	2.40 ± 0.51	2.31 ± 0.53	2.31 ± 0.52
f_B ($breaths \cdot min^{-1}$)	36 ± 8	33 ± 6	35 ± 7	35 ± 9
T_I/T_{TOT}	0.47 ± 0.01	0.48 ± 0.02	0.49 ± 0.04	0.48 ± 0.05
$[La^-]_B$ ($mmol \cdot L^{-1}$)	4.21 ± 1.88	$2.74 \pm 1.08^{*‡}$	$0.84 \pm 0.14^{§}$	0.92 ± 0.10
pH	7.357 ± 0.031	$7.376 \pm 0.020^{*†}$	$7.414 \pm 0.025^{§+}$	7.424 ± 0.022
PCO_2 (mmHg)	41.8 ± 3.5	41.4 ± 3.1	$41.9 \pm 3.8^{§§}$	40.9 ± 2.4
f_C ($beats \cdot min^{-1}$)	163 ± 15	$159 \pm 14^{*}$	$68 \pm 9^{+§§}$	68 ± 12
SpO_2 (%)	95 ± 1	95 ± 1	$99 \pm 1^{+§§}$	99 ± 1
RPE (Leg)	5.8 ± 1.3	$5.2 \pm 1.2^{**++}$	$0.0 \pm 0.0^{+§}$	0.0 ± 0.0
RPE (Dyspnoea)	3.9 ± 1.7	$2.0 \pm 1.5^{*++}$	$2.8 \pm 1.6^{§}$	1.7 ± 1.3
PLA				
$\dot{V}_E$ ($L \cdot min^{-1}$)	61.6 ± 14.5	59.6 ± 12.5	58.2 ± 13.4	59.1 ± 14.6
V_T (L)	1.77 ± 0.34	1.78 ± 0.27	1.69 ± 0.36	1.72 ± 0.40
f_B ($breaths \cdot min^{-1}$)	36 ± 7	35 ± 7	35 ± 7	35 ± 7
T_I/T_{TOT}	0.48 ± 0.01	0.48 ± 0.02	0.49 ± 0.03	0.49 ± 0.02
$[La^-]_B$ ($mmol \cdot L^{-1}$)	4.20 ± 1.19	4.00 ± 1.13	$0.98 \pm 0.18^{§++}$	0.93 ± 0.39
pH	7.358 ± 0.017	7.359 ± 0.022	$7.400 \pm 0.03^{§++}$	7.395 ± 0.03
PCO_2 (mmHg)	41.2 ± 2.7	41.1 ± 2.6	$42.1 \pm 2.8^{§}$	42.5 ± 3.2
f_C ($beats \cdot min^{-1}$)	158 ± 16	153 ± 14	$66 \pm 10^{+§§}$	63 ± 7
SpO_2 (%)	96 ± 1	96 ± 1	$99 \pm 1^{‡§}$	99 ± 1
RPE (Leg)	5.1 ± 2.7	5.2 ± 3.4	$0.0 \pm 0.0^{+§}$	0.0 ± 0.0
RPE (Dyspnoea)	3.7 ± 2.9	3.2 ± 4.4	$1.6 \pm 2.1^{+}$	1.3 ± 1.7

$\dot{V}_E$, minute ventilation; V_T, tidal volume; f_B, breathing frequency; T_I/T_{TOT}, duty cycle; $[La^-]_B$, blood lactate concentration; PCO_2, partial pressure carbon dioxide; f_C cardiac frequency; SpO_2, estimated arterial oxygen saturation; RPE, rating of perceived exertion. Significant difference [(from pre-intervention * $P < 0.01$, ** $P < 0.05$; intervention x time interaction † $P < 0.01$, †† $P < 0.05$); (between trial § $P < 0.01$, §§ $P < 0.05$; trial x time interaction $^{+}$ $P < 0.01$, $^{++}$ $P < 0.05$); (treatment x intervention x time interaction $^{‡}P < 0.05$)].

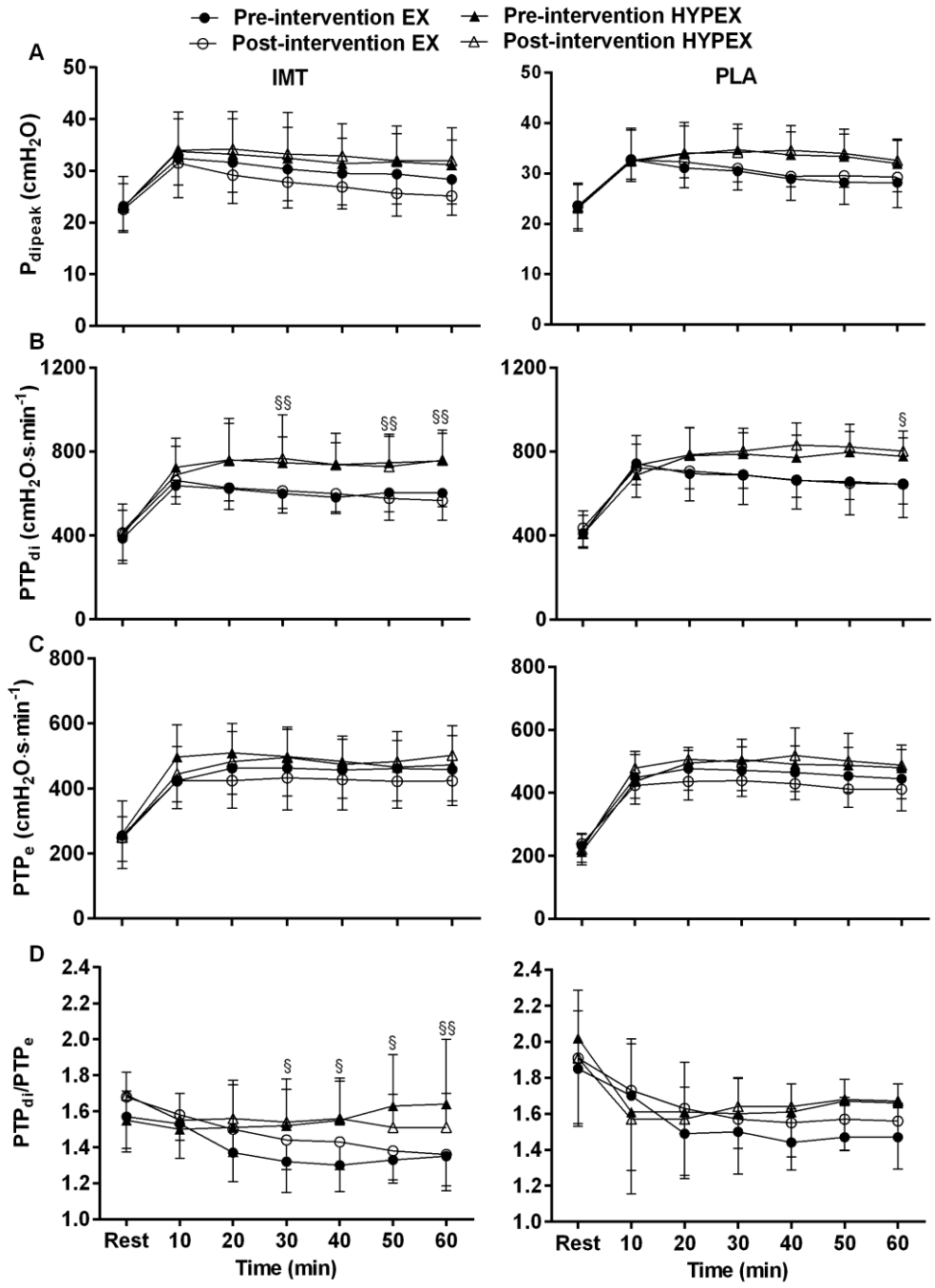


Figure 3.1 Peak transdiaphragmatic pressure (P_{dipeak}; A), diaphragm pressure-time product (PTP_{di}; B), inspiratory muscle pressure-time product (PTP_e; C) and the relative contribution of diaphragm to the inspiratory muscle pressure-time product (PTP_{di}/PTP_e; D) for IMT (left panels) and PLA (right panels) groups. Significant difference (between trial $^{\S}P < 0.01$, $^{\S\S}P < 0.05$). Values are mean ± SD.

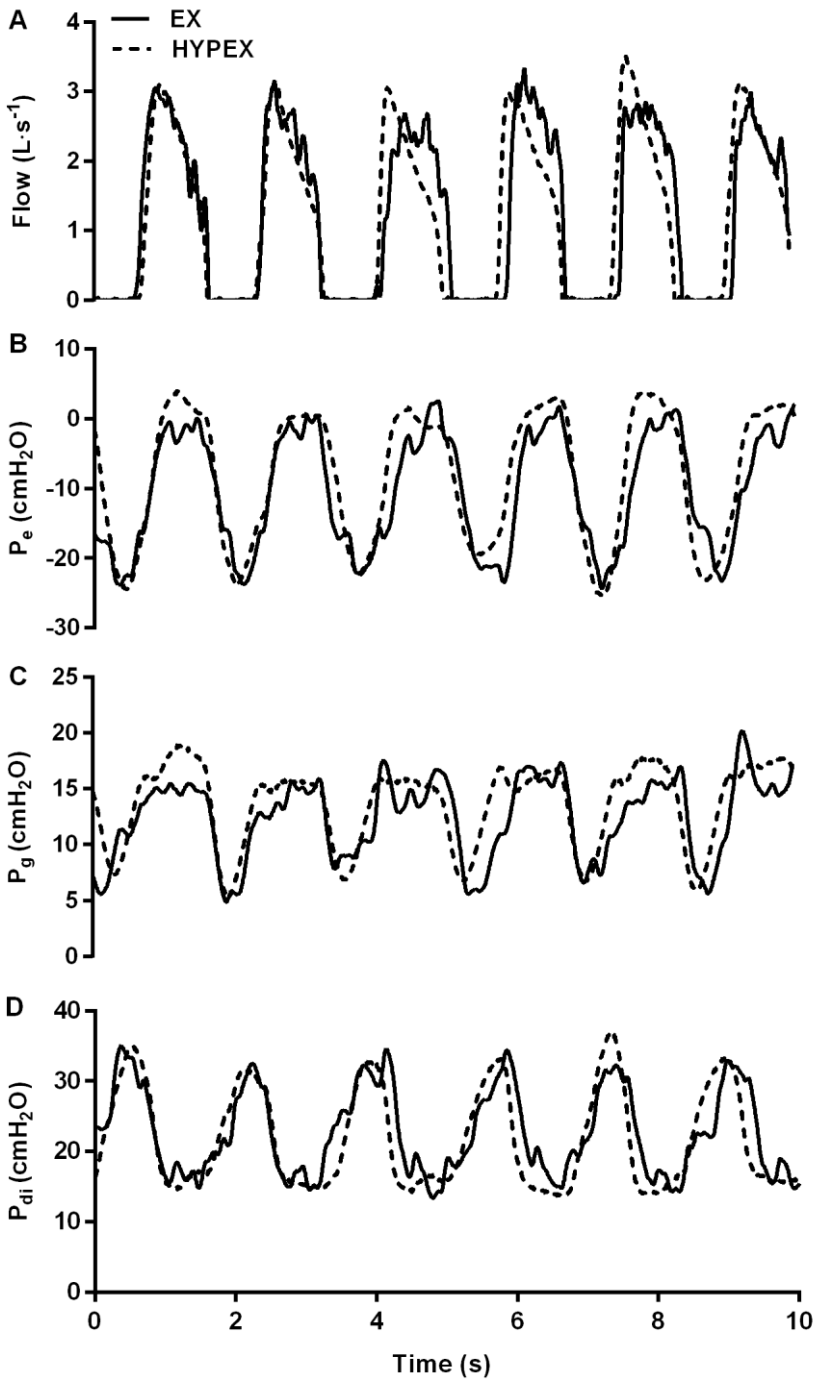


Figure 3.2 Representative trace of flow (A), oesophageal pressure (P_e; B), gastric pressure (P_g; C) and transdiaphragmatic pressure (P_{di}; D) for EX and HYPEX. Note that only expiratory flow is shown because the pneumotachograph was located on the expiratory port of the two-way non re-breathing valve.

3.2.3 PLASMA INTERLEUKIN-6 CONCENTRATION

Plasma IL-6 concentration peaked immediately after EX for both IMT and PLA groups and returned to resting values at +24 h (Figure 3.3). The plasma IL-6 response to EX was reduced by 33% following IMT (main effect for intervention, $P < 0.05$). Compared to rest, plasma IL-6 concentration increased in both groups during HYPEX (main effect for time, $P < 0.05$) and returned to resting values at +24 h. Increases in plasma IL-6 concentration during HYPEX were unchanged for both groups post-intervention, although there was a trend for an attenuated response following IMT (main effect for intervention, $P = 0.083$).

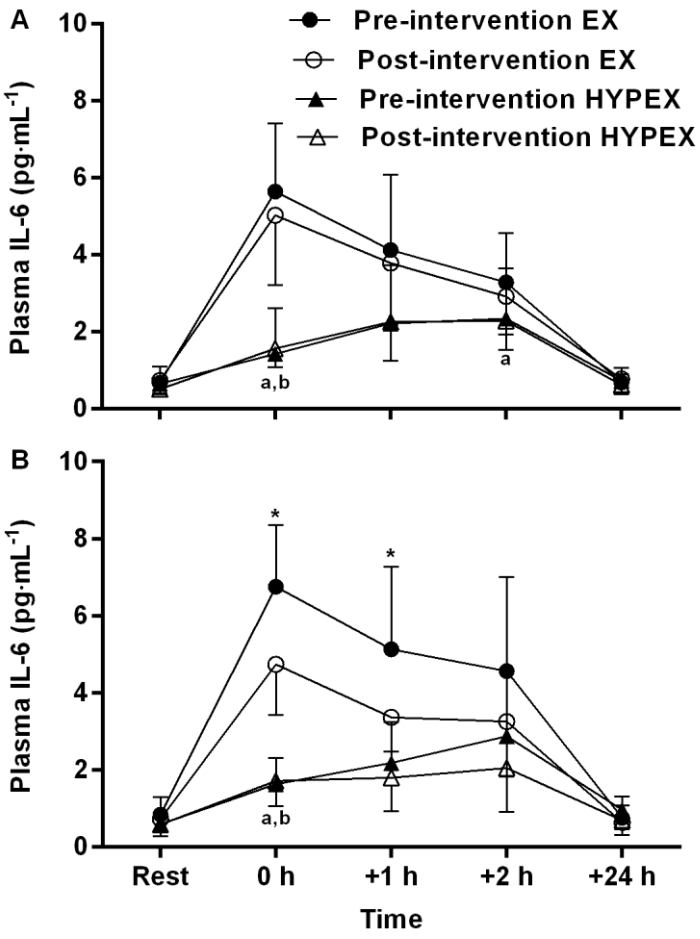


Figure 3.3 Plasma interleukin-6 (IL-6) concentration for PLA (A) and IMT (B) groups. Significant difference (from pre-intervention * $P < 0.05$; main effect time during HYPEX, $P < 0.01$ for [a] pre and [b] post-intervention). Values are mean ± SD.

3.2.4 BLOOD LACTATE CONCENTRATION AND BLOOD GASES

$[La^-]_B$ during EX was reduced by 35%, and pH was higher, following IMT (main effects for intervention, $P < 0.01$) (Table 3.2). Changes in PCO_2 during EX were unchanged in both groups post-intervention (Table 3.2). At rest, pre-intervention $[La^-]_B$ was 0.57 ± 0.11 and 0.76 ± 0.12 $mmol \cdot L^{-1}$ for IMT and PLA groups and increased during HYPEX for both groups (main effect for time, $P < 0.05$), and responses were unchanged post-intervention. pH and PCO_2 during HYPEX trials were maintained at rest for both groups.

3.2.5 TRANSDIAPHRAGMATIC TWITCH PRESSURES

A plateau (i.e., no significant increase in amplitude with increasing stimulation intensity) in M-wave and P_{ditw} amplitude (Figure 3.4) was observed in response to supramaximal BAMPS, indicating maximal depolarisation of the phrenic nerves. Non-potentiated P_{ditw} and potentiated P_{ditw} were unchanged following EX and HYPEX in both groups pre- and post-intervention (see Table 3.3 for measures taken at baseline and <15 min after EX and HYPEX; P_{ditw} and potentiated P_{ditw} did not change after <15 min) indicating there was no evidence of diaphragm fatigue. The inter- and intra-trial CV for baseline P_{ditw} and M-wave responses was <8%.

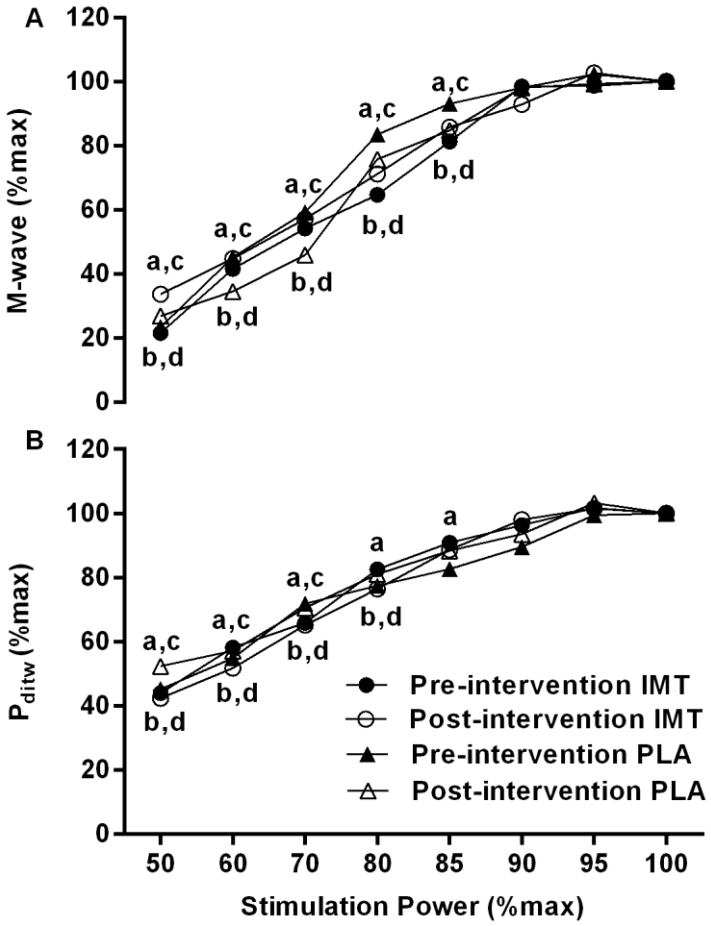


Figure 3.4 M-wave (A) and transdiaphragmatic twitch pressure (P_{ditw}; B) responses to supramaximal BAMPS for IMT and PLA groups. Values are pooled mean of PASSIVE, EX and HYPEX trials. Significant difference (from values at 100% of stimulators power output ($P < 0.05$) [a] pre-intervention IMT, [b] post-intervention IMT; [c] pre-intervention PLA, [d] post-intervention PLA). Values are mean.

Table 3.3 Transdiaphragmatic twitch pressures (P_{ditw}) at baseline and <15 min following EX and HYPEX for IMT and PLA groups. Values are mean ± SD.

	EX		HYPEX	
	Baseline	<15 min	Baseline	<15 min
Pre-intervention IMT				
P_{ditw} (cmH_2O)	15.6 ± 5.1	16.5 ± 5.1	14.4 ± 6.4	14.9 ± 7.2
Potentiated P_{ditw} (cmH_2O)	23.3 ± 8.6	24.7 ± 9.4	23.4 ± 9.5	21.3 ± 9.2
Post-intervention IMT				
P_{ditw} (cmH_2O)	16.3 ± 6.1	16.6 ± 5.0	14.8 ± 6.7	15.7 ± 6.2
Potentiated P_{ditw} (cmH_2O)	24.6 ± 8.4	25.1 ± 5.0	24.8 ± 9.3	22.6 ± 7.3
Pre-intervention PLA				
P_{ditw} (cmH_2O)	12.3 ± 2.5	14.0 ± 3.2	10.4 ± 2.4	10.1 ± 2.1
Potentiated P_{ditw} (cmH_2O)	18.7 ± 5.3	19.6 ± 4.6	16.9 ± 5.1	16.7 ± 5.0
Post-intervention PLA				
P_{ditw} (cmH_2O)	12.2 ± 2.3	14.0 ± 3.7	11.2 ± 2.6	12.1 ± 2.7
Potentiated P_{ditw} (cmH_2O)	19.6 ± 4.0	21.2 ± 3.7	17.6 ± 4.5	18.3 ± 4.9

3.2.6 PERCEPTUAL RESPONSES, CARDIAC FREQUENCY AND ARTERIAL OXYGEN SATURATION

Reductions in leg discomfort (main effect for intervention, $P < 0.05$) and dyspnoea (main effect for intervention, $P < 0.01$) were observed during EX after IMT (Table 3.2 and Figure 3.5). There was a trend for dyspnoea to be lower during HYPEX following IMT (main effect for intervention, $P = 0.054$). f_C was lower during EX following IMT (main effect intervention, $P < 0.05$). f_C and S_PO_2 responses during HYPEX were unchanged for both the IMT and PLA groups post-intervention (Table 3.2).

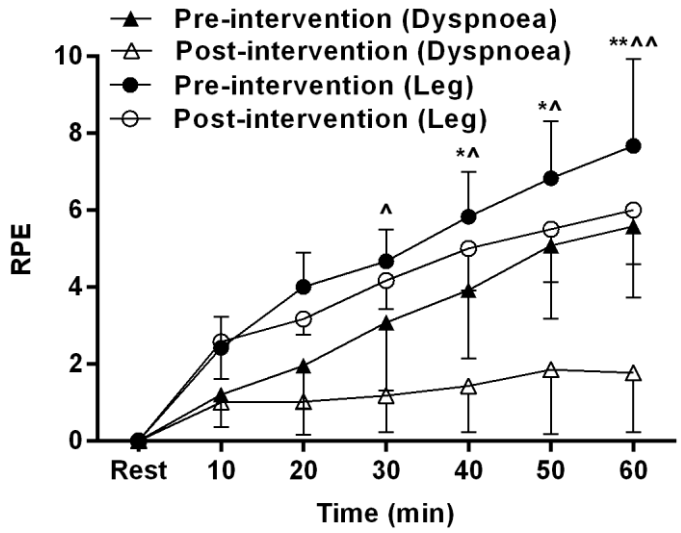


Figure 3.5 Rating of perceived exertion (RPE) for leg discomfort and dyspnoea for the IMT group during EX. Significant difference (from pre-intervention for leg ($^{*}P <$ 0.01, $^{**}P < 0.05$) and dyspnoea ($^{\wedge}P < 0.01$, $^{\wedge\wedge}P < 0.05$)). Values are mean ± SD.

3.3 DISCUSSION

3.3.1 MAIN FINDINGS

The main findings were that plasma IL-6 concentration increased following strenuous whole-body exercise (EX) and a volitional mimic of the breathing and respiratory muscle recruitment patterns achieved during EX (HYPEX), and that these increases occurred in the absence of diaphragm fatigue. Furthermore, IMT reduced the magnitude of the plasma IL-6 response to EX but not HYPEX.

To the author's knowledge, this is the first study to report an increase in plasma IL-6 concentration following a volitional mimic of the exercise hyperpnoea. The increase in IL-6 following HYPEX was of a similar magnitude to that reported following IRL at 75% MIP until task failure (Vassilakopoulos et al. 1999). However, the present experimental model provides a more ecologically valid approach to assess whether the respiratory muscles release IL-6 during whole-body exercise. Confidence that the increase in plasma IL-6 concentration observed during HYPEX

is not an artefact of the experimental conditions is evidenced by the PASSIVE trial, where plasma IL-6 concentration was unchanged. The present data therefore suggest that the respiratory muscles do indeed contribute to the increase in plasma IL-6 concentration observed during whole-body exercise.

3.3.2 STIMULI FOR INTERLEUKIN-6

The stimuli responsible for the increase in plasma IL-6 following HYPEX could not be established from the present study. There was no evidence of diaphragm fatigue following HYPEX, and confidence in the data is supported by the demonstration of supramaximal stimulation in M-wave and P_{ditw} amplitude and excellent inter/intra trial reliability in both. Therefore, the present data do not support the notion (Vassilakopoulos et al. 1999; Vassilakopoulos et al. 2002) that diaphragm fatigue is a likely trigger of IL-6 release.

Oxidative stress due to enhanced generation of ROS and nitrogen species is an important regulator of IL-6 production. ROS can upregulate IL-6 in the diaphragms of rats exposed to IRL (Sigala et al. 2011) and antioxidants can attenuate the plasma IL-6 response to IRL in humans (Vassilakopoulos et al. 2002). ROS are also upregulated by respiratory acidosis (Arbogast and Reid 2004); however, in the present study, PCO_2 was carefully maintained at resting levels. Upstream of ROS, NO production may be involved in the transcriptional signalling of IL-6 (Steensberg et al. 2007) and has been shown to regulate IL-6 production within the diaphragm of rats exposed to IRL (Sigala et al. 2012). A more likely mechanism for the production of IL-6 may be a reduction in respiratory muscle glycogen content. Muscle glycogen depletion during prolonged and strenuous lower-body exercise is a strong stimulus for enhanced IL-6 production by skeletal muscle fibres (Keller et al. 2005;

Steensberg et al. 2001). This notion is supported by the reduced diaphragm glycogen content of murines exposed to IRL (Ciufo et al. 2001) and whole-body exercise (Green et al. 1987; Ianuzzo et al. 1987).

3.3.3 SOURCE OF PLASMA INTERLEUKIN-6

Contracting skeletal muscle accounts for most (but not all) of the increase in plasma IL-6 observed during whole-body exercise (Steensberg et al. 2000). Thus, although the experimental model did not allow the source(s) of the increased plasma IL-6 during HYPEX to be established, the increase is attributed to, primarily, increased IL-6 release from contracting respiratory muscles. This notion is consistent with the increased IL-6 mRNA and protein expression in the diaphragm of rats exposed to IRL (Sigala et al. 2011; Sigala et al. 2012; Vassilakopoulos et al. 2004), and the higher IL-6 protein expression in the external intercostals of COPD patients compared to healthy controls (Casadevall et al. 2007).

However, there may have been additional contributions from other sources to the increase in plasma IL-6 concentration during HYPEX. Excessive expiratory muscle contractions during HYPEX (as evidenced by a higher PTP_{di}) may have resulted in greater IL-6 release from expiratory muscles. Excessive expiratory muscle contractions have been previously observed by those mimicking the pressure/flow characteristics of exercise hyperpnoea (Klas and Dempsey 1989). Other sources may include the brain that has been shown to release small amounts of IL-6 (~60 $pg \cdot min^{-1}$) following 60 min of cycling exercise (Nybo et al. 2002). Furthermore, IL-6 may be upregulated within the lungs and subsequently enter the systemic circulation from permeation of epithelial and endothelial barriers with increased transmural pressures (Uhlig 2002). This has been observed in murines exposed to negative or positive

pressure ventilated hyperventilation (von Bethmann et al. 1998) or IRL (Toumpanakis et al. 2010). However, this causes profound overdistension of the lung, which is unlikely during spontaneous hyperpnoea in humans due to self-regulation of end-inspiratory and/or end-expiratory lung volumes.

3.3.4 Effects of inspiratory muscle training on plasma interleukin-6 concentration

This is the first study to show that IMT reduces the plasma IL-6 response to whole-body exercise. Reduced IL-6 production by respiratory muscles following IMT may result from increases in basal levels of IL-6 rα (Akerstrom et al. 2009; Keller et al. 2005) or antioxidant enzymes (Yfanti et al. 2012), or a reduction in muscle glycogen utilisation (Croft et al. 2009; Fischer et al. 2004b) secondary to training-induced increases in mitochondrial density (Hoppeler and Fluck 2003).

However, the decrease in plasma IL-6 concentration during EX cannot be explained by this alone as: 1) plasma IL-6 concentration during HYPEX was not reduced; and 2) the reduction in plasma IL-6 concentration during EX following IMT was greater than the increase observed during HYPEX. Thus, an alternate explanation is that the decrease in f_C during EX could be evidence of a reduction in the respiratory muscle metaboreflex (Witt et al. 2007) which may serve to improve blood flow to the leg muscles (McConnell and Lomax 2006) or potentially the liver (Febbraio et al. 2003). Additional blood flow to the legs would increase oxygen (Bailey et al. 2010) and/or glucose delivery (Ebeling et al. 1993) potentially reducing muscle glycogen utilisation and blood flow to the liver would increase hepatosplanchnic uptake of IL-6 (Febbraio et al. 2003).

3.4 CONCLUSION

In conclusion, plasma IL-6 concentration is increased following strenuous whole-body exercise (EX) and a volitional mimic of the exercise hyperpnoea (HYPEX), and in the absence of diaphragm fatigue. Furthermore, IMT reduced the magnitude of the plasma IL-6 response to EX but not HYPEX. That the respiratory muscles may contribute to the increase in plasma IL-6 during whole-body exercise raises several key questions: what other inflammatory cytokines are released from respiratory muscles during exercise and what are the key regulators of this IL-6 release? Are other cytokines increased / decreased following IMT and does this create a pro- or anti-inflammatory response during exercise or volitional hyperpnoea?

CHAPTER 4 – INSPIRATORY MUSCLE TRAINING REDUCES PLASMA INTERLEUKIN-6 CONCENTRATION DURING A VOLITIONAL MIMIC OF HEAVY WHOLE-BODY EXERCISE HYPERPNOEA

4.0 INTRODUCTION

In Chapter 3 it was shown that the respiratory muscles can contribute to exercise-induced plasma IL-6 concentrations which occurred in the absence of diaphragm fatigue. What remains unknown from this finding is what other cytokines are released from the respiratory muscles during exercise, and what the key regulators of their release are.

Only very prolonged exercise with primarily an eccentric component results in a small increase in the plasma concentration of the pro-inflammatory cytokine IL-1β (Ostrowski et al. 1999). Therefore, it could be inferred that very prolonged and/or high levels of respiratory muscle work would be required to elicit a significant response in this cytokine; those similar to heavy whole-body exercise. The increase in plasma IL-6 concentration is also intensity dependent. With higher exercise intensities (thus more muscular work) there are greater increases in plasma IL-6 concentration (Scott et al. 2011). Whether respiratory muscle IL-6 is also intensity dependent is unknown. One of the roles of IL-6 is to stimulate the anti-inflammatory cytokine IL-1ra (Steenberg et al. 2003). IL-1ra acts to restrict and limit the extent of the inflammatory response by inhibiting signalling transduction of IL-1 (α and β) through the IL-1 receptor complex (Dinarello 2000). IL-1β acts to reduce muscle contractility and mediate muscle injury (Paulsen et al. 2012) and therefore the appearance of plasma IL-1β could be linked to the induction of diaphragmatic fatigue.

Oxidative stress also has a role in the aetiology of diaphragmatic fatigue. A decrease in muscle contractility has been observed *in vitro* by incubating unfatigued isolated diaphragm fibres with ROS (Lawler et al. 1997). Conversely, contractile performance is improved by administrating antioxidants (to scavenge ROS) before or

during fatiguing contractions *in vitro* (Diaz et al. 1994; Khawli and Reid 1994; Shindoh et al. 1992) and *in vivo* (Kelly et al. 2009b; Supinski et al. 1995; Supinski et al. 1997; Travaline et al. 1997b). In addition to playing a role in diaphragmatic fatigue, oxidative stress via ROS also upregulates the protein expression of IL-6 and IL-1β in the diaphragms of rats exposed to 6 h of IRL (Sigala et al. 2012).

In Chapter 3 it was also shown that IMT attenuated plasma IL-6 concentration during exercise but not a volitional mimic of the exercise hyperpnoea. Why this occurred is not known, but it was suggested that a change blood flow distribution (following IMT) during exercise played a role. It could also be speculated that the stimuli for the release of respiratory muscle IL-6 (i.e., oxidative stress, glycogen depletion) was not above a certain threshold to make any IMT-mediated adaptations evident during volitional hyperpnoea. Indeed, if glycogen depletion is a stimulus for IL-6 release from respiratory muscles then the respiratory muscles may have to undergo a certain amount of work before this is evident. There is no glycogen depletion in the diaphragms of rats after treadmill running at 63 ± 3% $\dot{V}O_{2max}$ whereas a 25% decrease is observed when they exercise at 84 ± 2% $\dot{V}O_{2max}$ (Fregosi and Dempsey 1986). Therefore, only respiratory muscle work associated with heavy exercise hyperpnoea may elicit a significant reduction in diaphragm glycogen content. Whole-body exercise training attenuates glycogen depletion and plasma IL-6 concentration (Croft et al. 2009; Fischer et al. 2004b) secondary to training-induced increases in mitochondrial density (Hoppeler and Fluck 2003). IMT may elicit similar morphological adaptations in the inspiratory muscles (Brown et al. 2008; Brown et al. 2010; Brown et al. 2012; Ramirez-Sarmiento et al. 2002), and thus also attenuate the plasma IL-6 response to the hyperpnoea associated with heavy whole-body exercise.

Therefore, the aim of this study was to use a volitional mimic of the breathing and respiratory muscle recruitment patterns associated with heavy whole-body exercise hyperpnoea and to examine whether the respiratory muscles contribute to exercise-induced increases in plasma IL-6, IL-1β and IL-1ra concentration and a systemic marker of oxidative stress (DNA damage levels in PBMC), if this release is related to diaphragm fatigue, and whether IMT attenuates the plasma IL-6 response associated with this higher level of respiratory muscle work.

4.1 METHODS

4.1.1 PARTICIPANTS

Twelve non-smoking recreationally active young males provided written, informed consent to participate in the study, which was approved by the Nottingham Trent University ethics committee (Appendix 1 and 2). Participants followed the pre-experimental instructions outlined in Section 2.0.

4.1.2 EXPERIMENTAL DESIGN

Participants attended the laboratory on 4 separate occasions, each separated by 48 h and at the same time of day, prior to a 6 week intervention. During the first visit participants were familiarised with all testing procedures and pulmonary function and MIP were measured. During the second visit participants performed a maximal incremental cycling test. The experimental trials were randomised, 1 h and comprised: (i) passive rest (PASSIVE) or (ii) volitional hyperpnoea at rest whereby participants mimicked the breathing and respiratory muscle recruitment patterns commensurate with heavy whole-body exercise (VH). Following the final trial participants were randomly, and equally, divided into an IMT or PLA group and

completed a 6 week intervention. At least 48 h after the intervention participants repeated the experimental trials in the same order as before the intervention. In all trials, participants either exercised or sat (in an identical position to the maximal incremental cycling test) on an electromagnetically braked cycle ergometer (Excalibur Sport; Lode).

4.1.3 PULMONARY FUNCTION AND MAXIMAL INSPIRATORY MOUTH PRESSURE

Pulmonary function and MIP were assessed according to the procedures stated in Sections 2.3 and 2.4, respectively.

4.1.4 MAXIMAL INCREMENTAL CYCLING TEST

Cycling began at 0 W and power was subsequently increased by 10 W every 15 s in order to result in exercise intolerance within ~10 min. This rapid incremental protocol was selected to maximise $\dot{V}_E$ at the cessation of the test and therefore reflect intense endurance exercise. The power at which exercise intolerance ensued defined $\dot{W}_{max}$, and the highest $\dot{V}O_2$ and $\dot{V}_E$ recorded in any 30 s period defined $\dot{V}O_{2peak}$ and $\dot{V}_{Epeak}$, respectively.

4.1.5 EXPERIMENTAL TRIALS

Trials were preceded by a 5 min rest period and during PASSIVE participants remained seated on the cycle ergometer for the duration of the trial. During VH participants mimicked at rest the breathing pattern ($\dot{V}_E$, f_B and T_I/T_{TOT}) and respiratory muscle recruitment pattern (P_{dipeak}) commensurate with 70–80% of the $\dot{V}_{Epeak}$ attained during the maximal incremental cycling test; which during pilot work was shown to represent the maximum square wave response that could be maintained

for 1 h. During the post-intervention VH trial participants mimicked the breathing and respiratory muscle recruitment patterns that were attained during the pre-intervention trial. These variables were ensemble averaged into six 10 min blocks from the pre-intervention trial and targets were updated during the post-intervention trial. An audio metronome paced f_B and T_I/T_{TOT}, and real-time visual feedback of $\dot{V}_E$ and P_{dipeak} was provided throughout. All experimental trials were performed in an environmental chamber (Design Environmental WIR52-20HS; Design Environmental) at 20°C and 90% relative humidity to minimise mucosal drying of the airways during VH.

4.1.6 FLOW, PULMONARY GAS EXCHANGE AND PRESSURE MEASUREMENTS

During all trials participants wore a facemask (model 7940; Hans Rudolph) connected to a pneumotachograph and ventilatory and pulmonary gas exchange responses were measured breath by breath (ZAN 600USB; Nspire Health). A two-way non-rebreathing valve (model 2730; Hans Rudolph) was attached distally to the pneumotachograph and on the expiratory port a Fleisch no. 3 pneumotachograph was attached and connected to a differential pressure transducer (± 2.5 cmH_2O) (TSD160A; BIOPAC Systems) and differential bridge amplifier (DA100C; BIOPAC Systems) to allow alignment of flow and pressure signals. P_e and P_g were measured according to the procedures stated in Section 2.6. P_{di} was calculated by subtracting P_e from P_g. P_{di} and P_e were integrated over the period of inspiratory flow and multiplied by f_B and labelled PTP_{di} and PTP_e, respectively. Non-physiological flows and pressures that resulted from swallowing, coughing and breath holding, were identified by visual inspection and removed.

4.1.7 BILATERAL ANTERIOR MAGNETIC PHRENIC NERVE STIMULATION

BAMPS was applied according to the procedures stated in Section 2.7. Non-potentiated P_{ditw} and potentiated P_{ditw} was measured at baseline and within 15 (<15 min), 35 (<35 min) and 60 (<60 min) min after each experimental trial. Diaphragm fatigue was defined as a >15% reduction in P_{ditw} compared to the baseline value (Kufel et al. 2002).

4.1.8 BLOOD ANALYSES AND ADDITIONAL MEASUREMENTS

Arterialised venous blood was sampled from a dorsal hand vein via an indwelling 21-G cannula (McLoughlin et al. 1992). During the experimental trials blood samples were taken at rest, every 3 min for the initial 15 min, and every 5 min thereafter, and analysed immediately for PCO_2 and pH (ABL520; Radiometer), which were corrected for changes in rectal temperature (Squirrel 2020; Grant Instruments). At rest and every 10 min during each trial $[La^-]_B$ was determined using an automated analyser (Biosen C_line Sport; EKF Diagnostics) and measurements were taken for f_C using short range telemetry (Polar S610; Polar), SpO_2 using infrared fingertip pulse oximetry (Model 8600; Nonin), and RPE for dyspnoea using Borg's modified CR10 scale (Borg 1982). At rest, immediately after (0 h) and 1 (+1 h), 2 (+2 h), and 24 (+24 h) h after each experimental trial, 3 x 5 mL blood samples were taken for the measurement of plasma cytokines. Participants were instructed to not undertake any physical activity between +2 h and + 24 h. Blood was transferred into pre-cooled tubes containing 1.6 $mg \cdot mL^{-1}$ of K_3E EDTA (SARSTEDT, Leicester, UK) and immediately centrifuged for 15 min at 1000 x *g* and 5ºC. The plasma supernatant was subsequently removed and stored at -80ºC until further analysis. Commercial solid phase sandwich ELISA were used to measure plasma

concentrations in duplicate of IL-6, IL-1β (Quantikine HS; R&D Systems) and IL-1ra (Quantikine; R&D Systems). The assays have a detection limit of 0.039 $pg \cdot mL^{-1}$ (IL-6), 0.1 $pg \cdot mL^{-1}$ (IL-1β) and 22 $pg \cdot mL^{-1}$ (IL-1ra), respectively. To minimise the effect of inter-assay variation, pre- and post-intervention cytokines from both groups were measured during the same assay. The inter/intra assay CV for ELISA were <10%.

4.1.9 MEASUREMENT OF SYSTEMIC OXIDATIVE STRESS

The comet assay was used to measure systemic oxidative stress by measuring levels of DNA damage in PBMC. During the experimental trials blood samples were taken at rest, immediately following the experimental trial (0 h) and 1 day into recovery (+24 h). Blood was immediately transferred into pre-cooled tubes containing lithium heparin (SARSTEDT). PBMC were isolated using density gradient centrifugation as stated in Section 2.13.1. The alkaline comet assay (Singh et al. 1988) and the modified alkaline comet assay (Collins et al. 1993) was used to determine levels of DNA damage (DNA single strand breaks and alkali labile lesions) in PBMC as stated in Section 2.13.2.

4.1.10 INSPIRATORY MUSCLE TRAINING AND PLACEBO INTERVENTIONS

The IMT group performed training sessions according to the procedures stated in Section 2.15. The PLA group inhaled one puff twice daily for 6 weeks from a sham metered dose inhaler (Vitalograph). The inhaler contained compressed air only but was promoted to the participants as delivering a novel drug that reduces breathing-induced inflammation. During the post-intervention period the IMT and PLA groups performed their intervention 2 $d \cdot wk^{-1}$, which is sufficient to maintain

improvements in inspiratory muscle function after IMT (Romer and McConnell 2003). These maintenance sessions were performed 48 h before and 48 h after experimental trials. All participants completed a training diary throughout the study to record adherence to the prescribed intervention and whole-body training sessions.

4.1.11 STATISTICAL ANALYSES

Statistical analyses were performed using SPSS for Windows (IBM). Groups were compared for pre-intervention characteristics using independent *t*-tests. A two-way repeated measures ANOVA was used to test between-group effects due to 'treatment' (IMT vs. PLA) and within-group effects due to 'intervention' (pre- vs. post-'treatment'), or 'time' (10, 20, 30, 40, 50 and 60 min for physiological variables; 0, +1 , +2 and +24 h for plasma cytokine data; 0 h and +24 h for DNA damage to PBMC data; or baseline, <15, <35 and <60 min for P_{ditw} data). Planned pairwise comparisons were made with repeated measures *t*-tests and with Bonferroni adjustment. Plasma cytokine and $[La^-]_B$ responses were also analysed using a three-way repeated measures ANOVA ('treatment' x 'intervention' x 'time'). Reliability was assessed using a CV calculated from a pooled mean of both groups and all trials. Pearson product-moment correlation coefficients were calculated to assess the relationship between selected variables. Statistical significance was set at $P < 0.05$. Results are presented as mean ± SD.

4.2 RESULTS

Age (IMT vs. PLA: 27 ± 7 vs. 22 ± 3 yr), body mass (80 ± 6 vs. 74 ± 7 kg), height (181 ± 5 vs. 177 ± 4 cm) and $\dot{V}O_{2peak}$ (52 ± 6.2 vs. 47 ± 5.7 $mL \cdot kg^{-1} \cdot min^{-1}$) were not different between groups. $\dot{W}_{max}$ (378 ± 21 vs. 336 ± 23 W) and $\dot{V}_{Epeak}$ (154 ±

19 vs. 120 ± 27 $L \cdot min^{-1}$) were higher ($P < 0.05$) for the IMT group. All other pre-intervention measurements were not different between groups. All responses remained unchanged from rest during PASSIVE trials.

4.2.1 PULMONARY FUNCTION AND MAXIMAL INSPIRATORY PRESSURE

Pre-intervention pulmonary function and MIP were within normal limits (Table 4.1). Compliance with the intervention was excellent in both IMT and PLA groups with 96 ± 1 and 93 ± 1% of sessions completed, respectively. Pulmonary function was unchanged in both groups post-intervention, whereas MIP increased by 24 ± 16% following IMT ($P < 0.01$). Inspection of training diaries revealed habitual whole-body training remained constant in both groups.

Table 4.1 Pulmonary function and maximal inspiratory pressure for IMT and PLA groups. Predicted values from Quanjer et al. (1993) and Wilson et al. (1984). Values are mean ± SD.

	IMT		PLA	
	Pre-intervention	Post-intervention	Pre-intervention	Post-intervention
FVC (L)	5.58 ± 0.44	5.65 ± 0.47	5.61 ± 0.67	5.49 ± 0.62
FVC (% Predicted)	104 ± 7	105 ± 5	105 ± 9	103 ± 8
FEV_1 (L)	4.49 ± 0.46	4.43 ± 0.20	4.53 ± 0.51	4.34 ± 0.57
FEV_1 (% Predicted)	100 ± 8	102 ± 7	100 ± 10	96 ± 12
FEV_1/FVC (%)	81 ± 6	81 ± 4	81 ± 6	80 ± 8
FEV_1/FVC (% Predicted)	99 ± 6	99 ± 5	98 ± 7	96 ± 9
MVV_{10} ($L \cdot min^{-1}$)	208 ± 2	206 ± 20	192 ± 33	196 ± 25
MVV_{10} (% Predicted)	111 ± 14	111 ± 11	102 ± 19	104 ± 14
MIP (cmH_2O)	168 ± 49	202 ± 39*	136 ± 32	139 ± 31
MIP (% Predicted)	157 ± 9	178 ± 34*	113 ± 26	115 ± 25

FVC, forced vital capacity; FEV_1, forced expiratory volume in 1 s; MVV_{10}, maximum voluntary ventilation in 10 s; MIP, maximal inspiratory pressure. Significant difference (from pre-intervention $^*P < 0.01$).

4.2.2 BREATHING AND RESPIRATORY MUSCLE RECRUITMENT PATTERN

Both IMT and PLA participants were able to accurately mimic the breathing and respiratory muscle patterns commensurate with heavy whole-body exercise hyperpnoea and replicated this accurately in their respective post-intervention VH trials. A representative breathing and respiratory muscle recruitment pattern trace is shown in Figure 4.1 and the targets for VH in Table 4.2. There were no differences in the breathing pattern (Table 4.3; PASSIVE data not shown) and respiratory muscle recruitment pattern (Figure 4.2) before or after the intervention for both groups. The mean $\dot{V}_E$ during the pre-intervention VH trial represented 71 ± 12 and 82 ± 6% of the $\dot{V}_{Epeak}$ attained during the maximal incremental cycling test for IMT and PLA groups, respectively. The inter-trial CV for P_{dipeak} at rest was <7%.

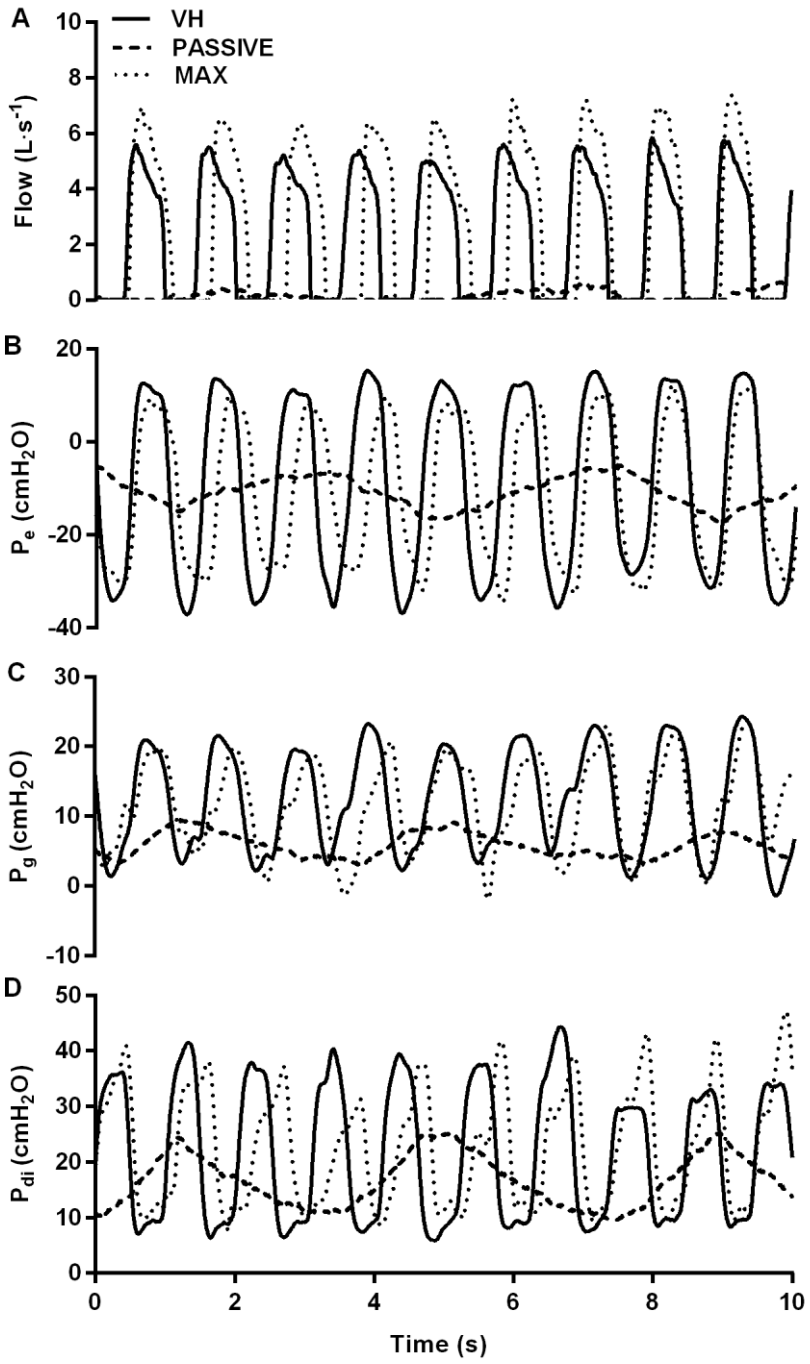


Figure 4.1 Representative trace of flow (A), oesophageal pressure (P_e; B), gastric pressure (P_g; C) and transdiaphragmatic pressure (P_{di}; D) for VH, PASSIVE and maximal incremental cycling (MAX). Note that only expiratory flow is shown because the pneumotachograph was located on the expiratory port of the two-way non re-breathing valve.

Table 4.2 Breathing and respiratory muscle recruitment pattern targets during VH for IMT and PLA groups. Values are mean ± SD.

	IMT	PLA
$\dot{V}_E$ ($L \cdot min^{-1}$)	126 ± 17	104 ± 17
f_B ($breaths \cdot min^{-1}$)	42 ± 10	37 ± 7
T_I/T_{TOT}	0.50 ± 0.02	0.51 ± 0.02
P_{dipeak} (cmH_2O)	35 ± 10	37 ± 8

$\dot{V}_E$, minute ventilation; V_T, tidal volume; f_B, breathing frequency; T_I/T_{TOT}, duty cycle; P_{dipeak}, peak transdiaphragmatic pressure.

Table 4.3 Physiological responses to VH for IMT and PLA groups.

	IMT		PLA	
	Pre-intervention	Post-intervention	Pre-intervention	Post-intervention
$\dot{V}_E$ ($L \cdot min^{-1}$)	109 ± 21	109 ± 16	99 ± 20	98 ± 21
V_T (L)	2.75 ± 0.73	2.71 ± 0.69	2.70 ± 0.47	2.66 ± 0.44
f_B ($breaths \cdot min^{-1}$)	42 ± 11	42 ± 11	37 ± 7	37 ± 6
T_I/T_{TOT}	0.49 ± 0.04	0.48 ± 0.05	0.50 ± 0.03	0.50 ± 0.02
$[La^-]_B$ ($mmol \cdot L^{-1}$)	1.37 ± 0.58	1.21 ± 0.40	1.29 ± 0.43	1.19 ± 0.47
pH	7.399 ± 0.043	7.409 ± 0.035	7.407 ± 0.028	7.408 ± 0.033
PCO_2 (mmHg)	44.4 ± 4.4	43.8 ± 4.2	41.3 ± 4.0	41.8 ± 4.5
f_C ($beats \cdot min^{-1}$)	84 ± 9	80 ± 10†	74 ± 6	74 ± 13
SpO_2 (%)	99 ± 1	99 ± 1	99 ± 1	99 ± 1

$\dot{V}_E$, minute ventilation; V_T, tidal volume; f_B, breathing frequency; T_I/T_{TOT}, duty cycle; $[La^-]_B$, blood lactate concentration; PCO_2, partial pressure carbon dioxide; f_C, cardiac frequency; SpO_2, estimated arterial oxygen saturation. Significant difference (intervention x time interaction † $P < 0.01$).

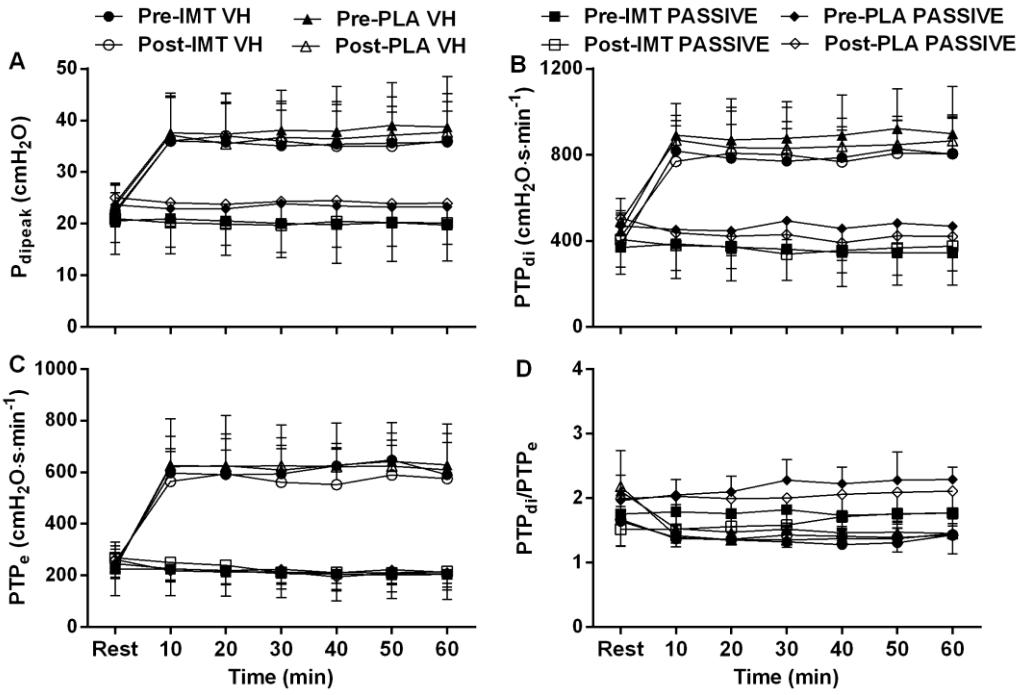


Figure 4.2 Peak transdiaphragmatic pressure (P_{dipeak}; A), diaphragm pressure-time product (PTP_{di}; B), inspiratory muscle pressure-time product (PTP_e; C) and the relative contribution of diaphragm to the inspiratory muscle pressure-time product (PTP_{di}/PTP_e; D) for IMT and PLA groups.

4.2.3 PLASMA CYTOKINE CONCENTRATIONS

Compared to rest, plasma IL-6 and IL-1β concentrations increased over time (main effect for time, $P < 0.05$) during pre- and post-intervention VH trials for both groups and returned to resting values at +24 h (Figure 4.3). Pairwise comparisons did not reveal any significant effects for plasma IL-1β. The plasma IL-6 response to VH was reduced by 24% (main effect for intervention, $P < 0.05$) following IMT whereas no changes were observed following PLA. No changes in the plasma IL-1β response to VH were observed post-intervention for both groups. Compared to rest, plasma IL-1ra concentrations did not increase over time during VH trials for both groups. Resting IL-1ra concentrations during the pre-intervention VH trial were 157 ± 46 and 172 ± 41 $pg \cdot mL^{-1}$ for the IMT and PLA groups, respectively.

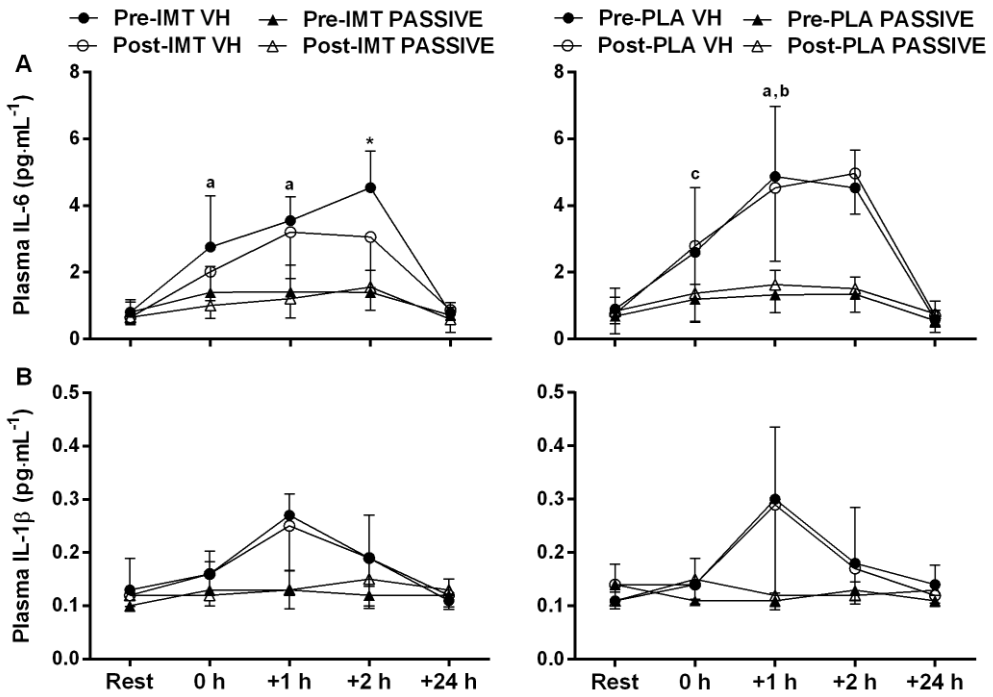


Figure 4.3 Plasma interleukin-6 (IL-6; A) and interleukin-1β (IL-1β; B) for the IMT (left panels) and PLA (right panels) groups. Significant difference [(from pre-intervention * $P < 0.05$); (main effect time during VH, $P < 0.01$ for ^{a} pre and ^{b} post-intervention; main effect time during VH, $P < 0.05$ for ^{c} pre-intervention)]. Values are mean ± SD.

4.2.4 DNA DAMAGE LEVELS IN PERIPHERAL BLOOD MONONUCLEAR CELLS

Compared to rest, the %DNA in Comet tail for the alkaline, FPG and ENDO III did not increase over time during VH trials before and after the intervention for both groups demonstrating that VH did not induce DNA damage in PBMC (Table 4.4) and systemic oxidative stress was not evident using this marker.

Table 4.4 DNA damage levels in peripheral blood mononuclear cells during VH for IMT and PLA groups.

	Rest	0 h	+24 h
Pre-intervention IMT			
Alkaline Comet (%DNA in Comet tail)	9.2 ± 4.6	7.2 ± 3.5	9.1 ± 5.2
FPG Comet (%DNA in Comet tail)	15.6 ± 6.3	13.6 ± 3.5	16.2 ± 9.8
ENDO III Comet (%DNA in Comet tail)	13.4 ± 5.5	13.0 ± 7.3	12.7 ± 5.5
Post-intervention IMT			
Alkaline Comet (%DNA in Comet tail)	7.3 ± 1.2	8.2 ± 3.3	8.8 ± 5.0
FPG Comet (%DNA in Comet tail)	16.9 ± 2.8	17.8 ± 7.3	17.3 ± 5.4
ENDO III Comet (%DNA in Comet tail)	11.4 ± 1.2	10.8 ± 2.0	9.8 ± 1.7
Pre-intervention PLA			
Alkaline Comet (%DNA in Comet tail)	14. 4 ± 10.0	9.8 ± 2.6	11.9 ± 7.1
FPG Comet (%DNA in Comet tail)	25.1 ± 7.4	21.9 ± 12.5	24.8 ± 8.1
ENDO III Comet (%DNA in Comet tail)	14.1 ± 8.6	13.7 ± 6.3	14.9 ± 8.8
Post-intervention PLA			
Alkaline Comet (%DNA in Comet tail)	11.2 ± 8.1	15.7 ± 9.0	11.8 ± 7.7
FPG Comet (%DNA in Comet tail)	21.6 ± 7.2	16.6 ± 11.7	25.0 ± 11.7
ENDO III Comet (%DNA in Comet tail)	15.6 ± 7.7	15.3 ± 7.9	15.1 ± 7.2

FPG, formamidopyrimidineglycosylase; ENDO III, endonuclease III.

4.2.5 TRANSDIAPHRAGMATIC TWITCH PRESSURES

A plateau (i.e., no significant increase in amplitude with increasing stimulation intensity) in M-wave and P_{ditw} amplitude (Figure 4.4) was observed in response to supramaximal BAMPS, indicating maximal depolarisation of the phrenic nerves. Non-potentiated P_{ditw} were unchanged following VH trials before and after the intervention for both groups. Compared to baseline responses, there was a reduction in potentiated P_{ditw} by 15–20% at <15 min post-VH (Figure 4.5), demonstrating evidence of diaphragm fatigue. This occurred in most VH trials, apart from post-intervention IMT trial, which was only 10%. Pairwise comparisons did not, however, reveal any significant effects. Pre-intervention increases in plasma IL-

6 and IL-1β during VH did not correlate with the decrease in P_{ditw}. The inter- and intra-trial CV for baseline P_{ditw} and M-wave responses was <7%.

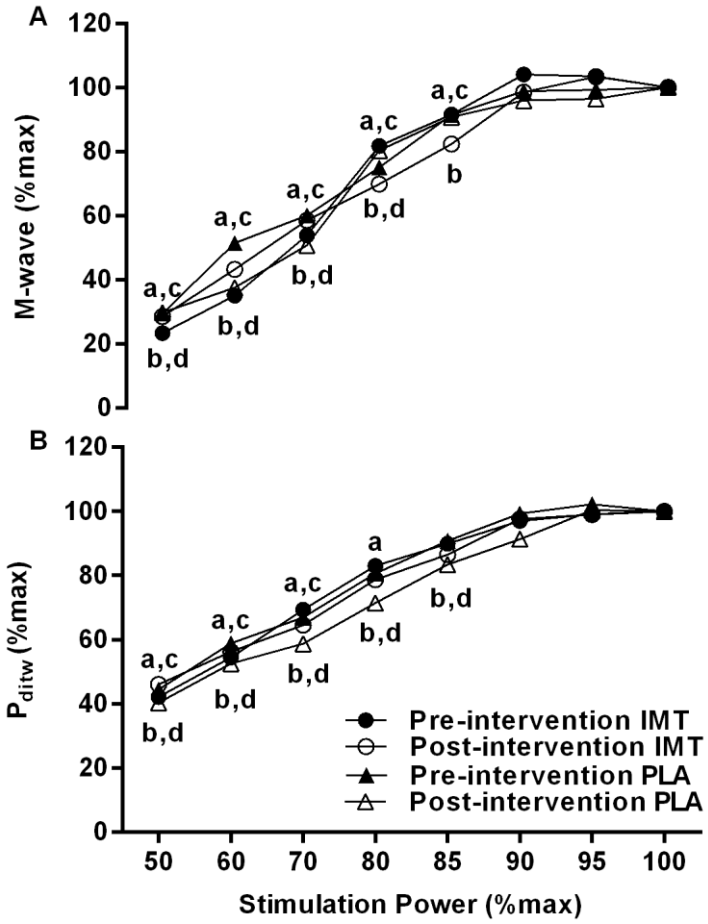


Figure 4.4 M-wave (A) and transdiaphragmatic twitch pressure (P_{ditw}; B) responses to supramaximal BAMPS for IMT and PLA groups. Values are pooled mean of VH and PASSIVE trials. Significant difference [from values at 100% of stimulators power output ($P < 0.05$ [a] pre-intervention IMT, [b] post-intervention IMT; [c] pre-intervention PLA, [d] post-intervention PLA)]. Values are mean.

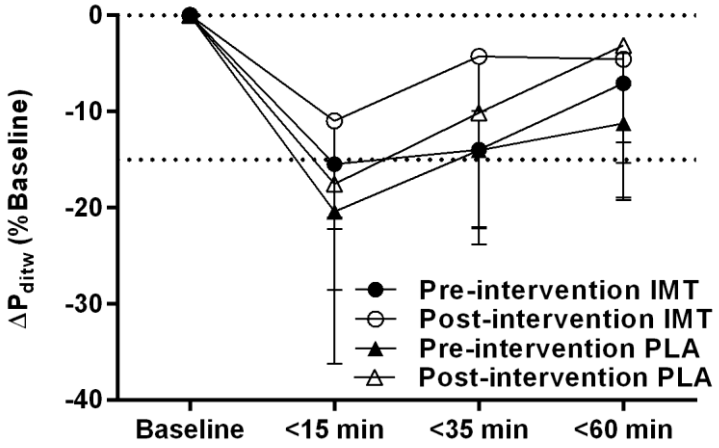


Figure 4.5 Potentiated transdiaphragmatic twitch pressure (P_{ditw}) responses to BAMPS at baseline and during recovery from VH for IMT and PLA groups. Values are mean ± SD of percentage baseline responses. Dashed lines represent the baseline (0%) and diaphragmatic fatigue (>15% reduction in P_{ditw} compared to the baseline values).

4.2.6 ADDITIONAL MEASUREMENTS

Compared to rest, $[La^-]_B$ increased over time (main effect for time, $P < 0.05$) and PCO_2, pH and SpO_2 were maintained at rest during VH trials for both groups (Table 4.3). There was no effect of the intervention on these measures in both groups. RPE dyspnoea was lower during VH (main effect for intervention, $P < 0.05$) and changed differently over time (intervention x time interaction, $P < 0.01$) following IMT whereas no changes were observed following PLA (Figure 4.6). The response of f_C was also different over time (intervention x time interaction, $P < 0.01$) following IMT whereas no changes were observed following PLA (Table 4.3).

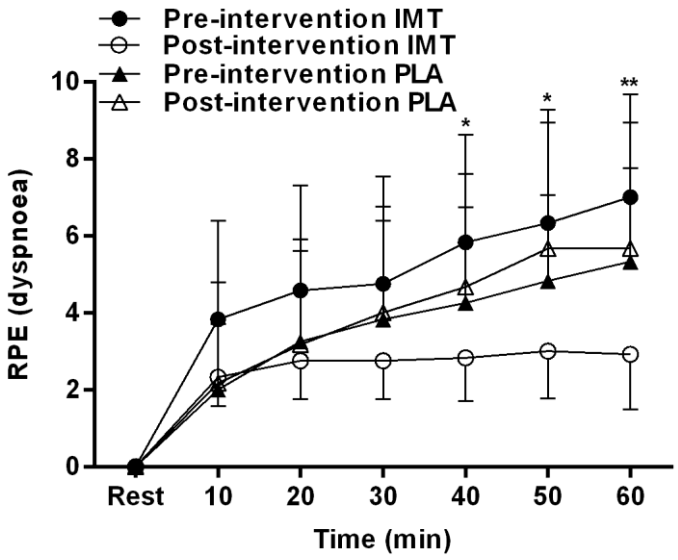


Figure 4.6 Rating of perceived exertion (RPE) for dyspnoea during VH for IMT and PLA groups. Significant difference (from pre-intervention for IMT group $^{**}P < 0.01$, $^{*}P < 0.05$).

4.3 DISCUSSION

4.3.1 MAIN FINDINGS

The main findings of this study were that plasma IL-6 and IL-1β concentrations increased following a volitional mimic of the breathing and respiratory muscle recruitment patterns achieved during heavy whole-body exercise (VH), and that these increases occurred with (but were not correlated to) diaphragmatic fatigue. There were no increases in the plasma IL-1ra concentration or DNA damage levels in PBMC after VH. Furthermore, IMT reduced the magnitude of the plasma IL-6 response to VH.

To the author's knowledge this is the first study the first to report an increase in plasma IL-6 and IL-1β concentration following a volitional mimic at rest of heavy exercise hyperpnoea. The increase in plasma IL-1β concentration following VH was of a similar magnitude to that reported following IRL at 75% MIP until task failure (Vassilakopoulos et al. 1999). The increase in plasma IL-6 concentration was,

however, much greater, and is similar to the peak concentrations observed after cycling exercise in Chapter 3. The increase in plasma IL-6 and IL-1β concentrations were not due to any methodological or environmental stresses since plasma IL-6 and IL-1β responses were unchanged from rest during PASSIVE.

4.3.2 STIMULI FOR INTERLEUKIN-6 AND INTERLEUKIN-1B

The stimuli responsible for the increase in plasma IL-6 and IL-β concentration following VH could not be established from the present study. Although there was evidence of diaphragm fatigue following VH this was not correlated with these markers. Therefore, our data do not support the notion (Vassilakopoulos et al. 1999; Vassilakopoulos et al. 2002) that diaphragm fatigue is a likely trigger of IL-6 or IL-1β release.

Oxidative stress due to enhanced generation of ROS and nitrogen species is an important regulator of IL-6 and IL-1β production. ROS can upregulate IL-6 and IL-1β in the diaphragms of rats exposed to IRL (Sigala et al. 2011) and antioxidants can attenuate the plasma IL-6 and IL-1β response to IRL in humans (Vassilakopoulos et al. 2002). ROS are also upregulated by respiratory acidosis (Arbogast and Reid 2004); however, PCO_2 in the present study was maintained at resting levels. Upstream of ROS, NO production may be involved in the transcriptional signalling of IL-6 (Steensberg et al. 2007) and has been shown to regulate IL-6 and IL-1β production within the diaphragm of rats exposed to IRL (Sigala et al. 2012). Although a marker of systemic oxidative stress (DNA damage in PBMC) was not increased following VH, it cannot be excluded that there was a local increase within the respiratory muscles. Alternatively, although others have observed an increase in markers of oxidative stress within the diaphragms of rats following

whole-body endurance exercise (Lawler et al. 1997; Lawler and Hu 2000; Oh-ishi et al. 1997), the current finding might suggest that this local production does not spill out to cause sufficient DNA damage in systemic PBMC.

A more likely mechanism for the production of IL-6 may be a reduction in respiratory muscle glycogen content. Muscle glycogen depletion during prolonged and strenuous lower-body exercise is a strong stimulus for enhanced IL-6 production by skeletal muscle fibres (Keller et al. 2005; Steensberg et al. 2001). This notion is supported by the reduced diaphragm glycogen content of murines exposed to IRL (Ciufo et al. 2001) and whole-body exercise (Green et al. 1987; Ianuzzo et al. 1987).

4.3.3 Source of plasma interleukin-6 and interleukin-1β

Although the current experimental model did not allow the source(s) of the increased plasma IL-6 and IL-1β concentration during VH to be established, this increase is attributed to, primarily, increased release from contracting respiratory muscles. This notion is consistent with the increased IL-6 and IL-1β mRNA and protein expression in the diaphragm of rats exposed to IRL (Sigala et al. 2011; Sigala et al. 2012; Vassilakopoulos et al. 2004), and the higher IL-6 and IL-1β protein expression in the external intercostals of COPD patients compared to healthy controls (Casadevall et al. 2007).

However, there may have been additional contributions from other sources to the increase in plasma IL-6 and IL-1β concentration during VH. The brain has been shown to release small amounts of IL-6 (~60 $pg \cdot min^{-1}$) following 60 min of cycling exercise (Nybo et al. 2002). Furthermore, IL-6 and IL-1β may be upregulated within the lungs and subsequently enter the systemic circulation from permeation of epithelial and endothelial barriers with increased transmural pressures (Uhlig 2002).

This has been observed in murines exposed to negative or positive pressure ventilated hyperventilation (von Bethmann et al. 1998) or IRL (Toumpanakis et al. 2010). However, this causes profound overdistension of the lung, which is unlikely during spontaneous hyperpnoea in humans due to self-regulation of end-inspiratory and/or end-expiratory lung volumes.

4.3.4 ROLE OF DIAPHRAGM FATIGUE

Low frequency diaphragm fatigue was observed following VH. This may have occurred because of the high levels of respiratory muscle work, causing amongst other things, an accumulation of metabolites, excessive production of ROS and/or glycogen depletion. Others have observed diaphragm fatigue following short-duration and very intense volitional hyperpnoea (Babcock et al. 1995; Hamnegard et al. 1996; Mador et al. 1996). The induction of fatigue was not related to plasma IL-6 or IL-β concentrations and it cannot be confirmed what the cause of it was. Based on the observation of the substantial increases in plasma IL-6 concentration, and the duration of VH, it is suggested that this may be due to glycogen depletion. Supplemented with the observation of an increase in plasma IL-1β, the findings may indicate that the diaphragm was injured in response to VH. Markers of respiratory muscle injury have been observed in humans following IRL at ~70% MIP for 1 h (Foster et al. 2012) which is another protocol performed at rest that significantly increases respiratory muscle work, and when strenuous enough can elicit diaphragmatic fatigue (Laghi et al. 1995; Laghi et al. 1996; Mador et al. 1996; Moxham et al. 1981; Sheel et al. 2001; Sheel et al. 2002; Travaline et al. 1997a; Travaline et al. 1997b).

4.3.5 PLASMA IL-1 RECEPTOR ANTAGONIST

Why there was not an increase in plasma IL-1ra is unknown. The only known biological role of IL-1ra is to inhibit signalling transduction of IL-1 through the IL-1 receptor complex (Dinarello 2000). It is has been suggested that IL-1ra is also upregulated by IL-6 and that acute exercise induces an anti-inflammatory environment in the post-exercise period (Steensberg et al. 2003). Therefore, theoretically, the increases in plasma IL-6 and IL-1β should lead to an increase in plasma IL-1ra. The likely explanation for this finding is the time course of plasma IL-6 and IL-1β in the present study. Following prolonged and strenuous whole-body exercise others have reported a plasma IL-1ra increase similar to the magnitude of the plasma IL-6 response, but with peak concentrations recorded between 2 to 4 h post-exercise (Ostrowski et al. 1999; Scott et al. 2011; Yfanti et al. 2012). The peak concentrations of plasma IL-6 and IL-1β in the present study were observed at +2 h and +1 h, respectively, which is consistent with IRL (Vassilakopoulos et al. 2002), but in contrast to whole-body exercise where these cytokines peak immediately post-exercise (Ostrowski et al. 1999). It is probable that this delay, which was accompanied by relatively low concentrations of plasma IL-6 and IL-1β (compared to prolonged and strenuous exercise bouts such as a marathon) resulted in plasma IL-1ra not being observed in the systemic circulation until ~2 h following this peak concentration; possibly at ~3 to 4 h post-VH and at a time point that was not measured.

4.3.6 EFFECTS OF INSPIRATORY MUSCLE TRAINING ON PLASMA INTERLEUKIN-6 CONCENTRATION

This is the first study to show that IMT reduces the plasma IL-6 response to volitional hyperpnoea. Reduced IL-6 production by respiratory muscles following

IMT may result from increases in basal levels of IL-6 rα (Akerstrom et al. 2009; Keller et al. 2005) or antioxidant enzymes (Yfanti et al. 2012), or a reduction in muscle glycogen utilisation (Croft et al. 2009; Fischer et al. 2004b) secondary to training-induced increases in mitochondrial density (Hoppeler and Fluck 2003). The hypothesis that a certain level of glycogen depletion may be required in the respiratory muscles to make any IMT-mediated adaptations evident during volitional hyperpnoea would be supported by this study and infer that if glycogen depletion is a stimulus for IL-6 release from respiratory muscles, then the respiratory muscles may have to undergo a certain amount of work before the effect of IMT is evident.

4.4 CONCLUSION

In conclusion, plasma IL-6 and IL-1β concentrations are increased following a volitional mimic of the breathing and respiratory muscle recruitment patterns achieved during heavy whole-body exercise (VH), and these increases occurred with (but were not correlated to) diaphragm fatigue. There were no increases in plasma IL-1ra concentration or DNA damage levels in PBMC after VH. Furthermore, IMT reduced the magnitude of the plasma IL-6 response to VH.

CHAPTER 5 – INSPIRATORY MUSCLE FUNCTION AND STRUCTURE ARE IMPROVED BUT RESTING SYSTEMIC MARKERS OF INFLAMMATION AND OXIDATIVE STRESS REMAIN UNCHANGED IN OLDER ADULTS FOLLOWING INSPIRATORY MUSCLE TRAINING

5.0 INTRODUCTION

In Chapters 3 and 4 it was shown that increased respiratory muscle work increases plasma cytokine concentrations. The respiratory muscles of older adults also experience increased workloads at rest. With healthy ageing there is a progressive decrease in total respiratory system compliance, which results in airflow limitation, air trapping, and an increase in residual volume (Vaz Fragoso and Gill 2012). The increase in residual volume flattens the curvature of the diaphragm, shifting its length-tension relationship to a shorter length and placing the diaphragm at a mechanical disadvantage (Polkey et al. 1998). This contributes to a reduction in inspiratory muscle force and endurance whilst increasing the O_2 cost of breathing (Harik-Khan et al. 1998; Johnson et al. 1997; Polkey et al. 1997; Takishima et al. 1990). Functionally, this can increase dyspnoea during everyday tasks, limit exercise capacity, and lead to reduced PAL and QoL (Jensen et al. 2009; Renwick 2001).

RMT in the forms of pressure-threshold IMT, combined pressure-threshold IMT/EMT and VIH have been promoted as a tool to attenuate the age-related decline in respiratory function. Manufacturers of devices have claimed that in older adults RMT can "increase resistance to fatigue", "improve respiratory muscle efficiency" and "alleviate dyspnoea" all resulting in "improved exercise capacity" (http://www.powerbreathe.com/breathing-facts). Indeed, several studies have supported these claims and reported increases in respiratory muscle strength and endurance, pulmonary function, exercise capacity, PAL and QoL (Aznar-Lain et al. 2007; Belman and Gaesser 1988; Huang et al. 2011; Watsford and Murphy 2008). Nevertheless, these studies have risked participant bias by failing to employ a placebo group (Belman and Gaesser 1988; Huang et al. 2011; Watsford and Murphy

2008) or are limited by a small sample size with insufficient statistical power (Aznar-Lain et al. 2007).

Healthy ageing is also associated with systemic low-level inflammation due to enhanced production of pro-inflammatory cytokines (Bruunsgaard 2006; Kregel and Zhang 2007). Although a marker of systemic oxidative stress (DNA damage in PBMC) was not increased during elevated respiratory muscle work in Chapter 4, markers of oxidative stress are significantly higher in the external intercostals of older compared to younger adults (Barreiro et al. 2006), suggesting that the respiratory muscles of older adults may be prone to oxidatively mediated damage to biomolecules such as nucleic acids (Barnett and Barnett 1998; King and Barnett 1995). Systemic inflammation may contribute to morbidity and mortality in ageing from hyperglycaemia, insulin resistance and increased gluconeogenesis (Beavers et al. 2010) and an elevation in bone reabsorbtion (Aris et al. 1998; Smith et al. 2008), dyslipidemia (Marik 2006), and proteolysis (Mitch and Goldberg 1996) whilst also increasing other parts of the inflammatory cascade that can have downstream biological effects. In Chapters 3 and 4 it was shown that IMT can attenuate plasma IL-6 concentrations during increased respiratory muscle work. In older adults, it has been reported that whole-body resistance or endurance training can decrease resting systemic cytokine concentrations (Kohut et al. 2006; Nicklas et al. 2008; Phillips et al. 2010; Prestes et al. 2009) and markers of oxidative stress (Fatouros et al. 2004; Parise et al. 2005; Takahashi et al. 2012), suggesting that regular conditioning of skeletal muscles may have a therapeutic benefit in attenuating these markers. Whether conditioning of the inspiratory muscles using IMT can have the same effect is unknown.

Therefore, the aim of this study was to examine the effects of IMT in older adults using a rigorous placebo controlled design, with an adequate sample size, on markers of systemic inflammation (plasma cytokines) and oxidative stress (DNA damage in PBMC) and to re-examine the claimed benefits of IMT on inspiratory muscle strength and endurance, pulmonary function, exercise capacity using the 6MWT, PAL and QoL. A further aim was to evaluate the effects of IMT on diaphragm thickness and dynamic inspiratory muscle function which is shown to increase in younger adults following IMT (Downey et al. 2007; Romer and McConnell 2003).

5.1 METHODS

5.1.1 PARTICIPANTS

A power analysis demonstrated that for the intervention to reduce a resting plasma IL-6 concentration of 2.5 ± 0.5 pg·mL^{-1} by 0.5 pg·mL^{-1}, 16 participants were required for each IMT and PLA group. The power analysis was based on the reductions observed following IMT in Chapters 3 and 4. Participants (65−75 years) were recruited in response to local advertising according to the exclusion criteria used to define "healthy" (i.e., disease free non-smokers and not on any daily medication including antioxidants or supplements) older participants for exercise studies (Appendix 4; Greig et al. 1994). In total, 38 participants were screened for suitability and provided written, informed consent to participate in the study, which was approved by the Nottingham Trent University ethics committee (Appendix 5). Of these, one was excluded due to a FEV_1 of <70% of predicted values (Falaschetti et al. 2004) and another developed bronchitis before commencement of the experimental trials. Each participant completed a 24 h estimated diet record prior to

their first experimental trial, which was then replicated prior to subsequent trials. Throughout the study, participants were instructed to adhere to their habitual training regimen and not to engage in any strenuous exercise the day preceding and the day of a trial. Participants arrived at the laboratory after an overnight fast (morning visits) or 4 h postprandially (afternoon visits) having abstained from alcohol and caffeine in the 24 h before testing.

5.1.2 EXPERIMENTAL DESIGN

A randomised, placebo-controlled design was used. Prior to randomisation, all participants undertook a screening session, where a full 12-lead electrocardiogram was performed and subsequently interpreted by a trained cardiologist (Appendix 6). Height, body mass, blood pressure, pulmonary function and maximal mouth pressures were also measured. For the experimental trials, participants attended the laboratory on two separate occasions before and after an 8 week intervention, each separated by at least 1 week and at the same time of day. During the first visit, participants were familiarised with all testing procedures and pulmonary function and maximal mouth pressures were measured. Participants also completed a questionnaire to determine QoL and were given an accelerometer to measure quantitative PAL. During the second visit, participants returned the accelerometer and a blood sample was taken and body fat, diaphragm thickness, dynamic inspiratory muscle function and inspiratory muscle endurance were assessed and participants performed a 6MWT. In addition, participants completed a questionnaire to measure qualitative PAL. Participants were then randomly, and equally, divided into an IMT or PLA group. Following the intervention, participants repeated the experimental trials in the same order. Results from the experimental trials only were

used in the subsequent analysis. In addition to improving participants' safety, the screening and familiarisation session(s) were used to minimise any learning effect and improve the reliability of the measurements (Eastwood et al. 1998; Kervio et al. 2003; McConnell and Copestake 1999; Polkey and Moxham 2004).

5.1.3 PULMONARY FUNCTION AND MAXIMAL INSPIRATORY AND EXPIRATORY MOUTH PRESSURE

Pulmonary function and maximal mouth pressures were assessed according to the procedures stated in Sections 2.3 and 2.4, respectively.

5.1.4 DIAPHRAGM THICKNESS

Diaphragm thickness (T_{di}) was assessed using B-mode ultrasonography (Phillips ATL HDI 5000; ATL Ultrasound, Washington, USA) according to current recommendations (ATS/ERS 2002). Participants were semi-recumbent and the eighth and ninth intercostal spaces in the right midaxillary line were identified and marked with a wax pencil. The transducer (Linear array 5−12 MHz probe; ATL Ultrasound) was held perpendicular to the chest wall and a two-dimensional coronal image of the diaphragm at the zone of apposition was identified by 2 clear parallel echodense lines (Figure 5.1). T_{di} was measured from the middle of the pleural and peritoneal membranes. Images were recorded via a personal computer interfaced with the ultrasound scanner and subsequently digitised at 25 Hz and analysed using a public domain software package (ImageJ; National Institutes of Health, Maryland, USA).

Measurements of T_{di} were obtained in triplicate (Figure 5.1) at residual volume ($T_{di.RV}$), functional residual capacity ($T_{di.FRC}$), and total lung capacity ($T_{di.TLC}$) and during a Müeller manoeuvre from residual volume ($T_{di.CONT}$). The average value

from each measurement was used in the analysis. In order to standardise for any changes in lung volume post-intervention and, therefore, obtain measurements with the diaphragm in a more contracted state, the diaphragm thickening ratio ($T_{di.TR}$) was determined using the formula: $T_{di.RV}$ / $T_{di.CONT}$. Lung volumes were estimated from flow signals using the pneumotachograph setup described in Section 5.1.5. Ultrasound images were synchronised with flow signals using a custom-built trigger.

To increase reproducibility and accuracy of the measurement, the same investigator completed each diaphragm assessment and was diligent in ensuring the probe was at the same alignment and internal markers were observed before and after the intervention. A semi-permanent mark was placed on each participant at the level of the probe and re-marked for each laboratory visit.

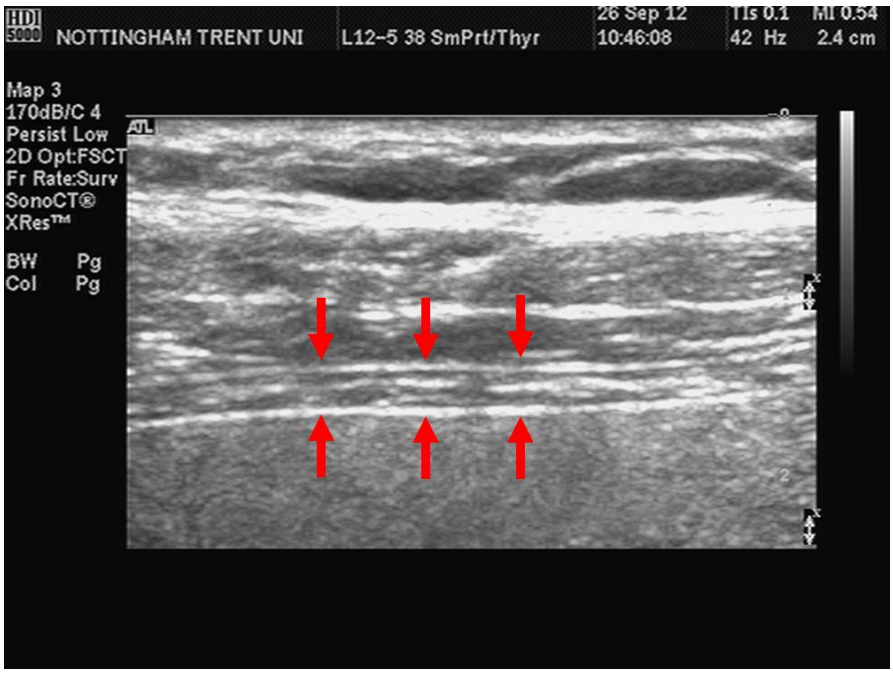


Figure 5.1 Example of diaphragm thickness measurement at functional residual capacity ($T_{di.FRC}$) in a male participant at pre-intervention. The red arrows mark both the peritoneal (top) and pleural (bottom) membranes of the diaphragm. Mean ± SD of $T_{di.FRC}$ measurements was 2.3 ± 0.1 mm.

5.1.4.1 REPRODUCIBILITY OF DIAPHRAGM THICKNESS MEASUREMENTS

Five healthy males and one female with normal respiratory function visited the laboratory on two separate visits and T_{di} measurements were undertaken using the procedure described above. Their age, body mass and height (mean ± SD) were 36 ± 14 years, 75 ± 12 kg and 174 ± 10 cm, respectively. Reproducibility was assessed using the CV.

The between-occasion reproducibility of the T_{di} measurements is shown in Table 5.1 and apart from $T_{di.TR}$ was excellent for all other measures. The calculation of $T_{di.TR}$ uses a Müeller manoeuvre which is effort dependent and may hinder reproducibility.

Table 5.1 Reproducibility of diaphragm thickness (T_{di}) measurements. Values are mean ± SD.

	Visit 1	Visit 2	CV
$T_{di.FRC}$ (mm)	2.01 ± 0.23	2.11 ± 0.14	7.01
$T_{di.TLC}$ (mm)	4.30 ± 0.66	4.27 ± 0.64	2.22
$T_{di.TR}$	2.46 ± 0.47	2.0 ± 0.44	14.2

CV, coefficient of variation; $T_{di.FRC}$, functional residual capacity; $T_{di.TLC}$, total lung capacity; $T_{di.TR}$, thickening ratio.

5.1.5 MAXIMAL DYNAMIC INSPIRATORY MUSCLE FUNCTION

Maximal dynamic inspiratory muscle function (Romer et al. 2002a; Romer and McConnell 2003) was assessed by performing maximal inspiratory efforts against a weighted plunger threshold device that housed two air-flow ports (Figure 5.2; Johnson et al. 1996; Nickerson and Keens 1982). On the first air-flow port, a two-way non-rebreathing valve (model 500975; Harvard Apparatus, Kent, UK) was attached with a 1 m length of wide-bore tubing connected. Inspiratory mouth pressure was measured using 14-G needle blunt inserted through a 2 mm aperture

located near the mouthpiece that was attached to a differential pressure transducer (± 400 cmH_2O) (TSD104A; BIOPAC Systems) and a differential bridge amplifier (DA100C; BIOPAC Systems). Inspiratory flow was measured using a Fleisch no. 3 pneumotachograph connected to a differential pressure transducer (± 2.5 cmH_2O) (TSD160A; BIOPAC Systems) and differential bridge amplifier (DA100C; BIOPAC Systems) that was connected to the other air-flow port of the weighted plunger threshold device.

Maximal inspiratory pressure at zero flow (P_{0max}) was measured by placing a rubber bung distal to the pneumotachograph and exposing a 1 mm leak to prevent glottic closure. Subsequently, participants performed 3 inspiratory efforts separated by 30 s in random order at approximately 0, 20, 25, 35, 50 and 65% P_{0max}. Participants wore a noseclip and manoeuvres were performed whilst standing, initiated from residual volume, and they received both visual and verbal feedback of voluntary efforts. Participants were encouraged to inspire as fast and as hard as possible. Pressure and flow fatigue is negligible following this protocol (Romer et al. 2002a; Romer and McConnell 2003).

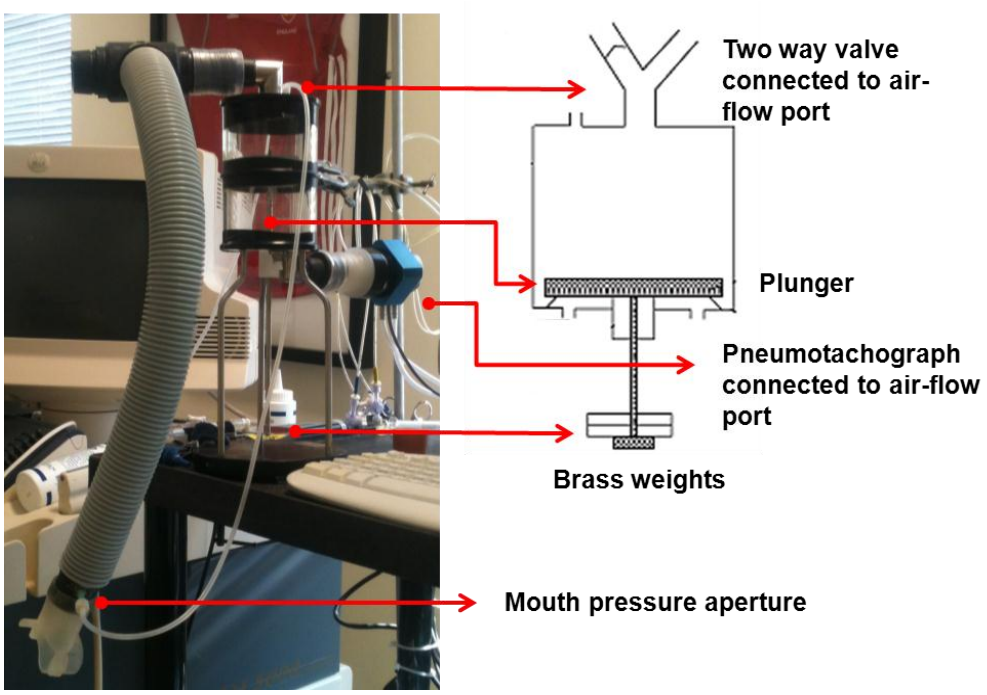


Figure 5.2 Weighted plunger inspiratory pressure-threshold device (Johnson et al. 1996).

The maximal value recorded for both inspiratory pressure (P_0) and inspiratory flow ($\dot{V}_I$) at each $\%P_0$ was used for analysis of which the product defined inspiratory muscle power ($\dot{W}_I$). Maximal $\dot{V}_I$ ($\dot{V}_{Imax}$) was calculated by extrapolation of a linear least squares representation of the $P_0 - \dot{V}_I$ data. A 2nd order polynominal was fitted to the $\dot{W}_I - \dot{V}_I$ data and maximal $\dot{W}_I$ ($\dot{W}_{Imax}$) was calculated by differentiation of the quadratic equation. Optimal inspiratory pressure (P_{opt} and $\%P_0 = P_{opt} / P_0$) and optimal $\dot{V}_I$ ($\dot{V}_{opt}$ and $\%\dot{V}_{max} = \dot{V}_{opt} / \%\dot{V}_{max}$) were defined as the pressure and flow values corresponding to the asymptote of the $\dot{W}_I$ curve ($\dot{W}_{Imax}$) and the point at which this vertically transected the linear representation of the pressure-flow relationship. A schematic for the pressure-flow-work calculations for the IMT group at pre-intervention is shown in Figure 5.3. Maximal rate of inspiratory pressure development (MRPD) was assessed during an inspiratory effort at P_{0max}. MRPD was defined as the positive peak of the pressure derivative as a function of time or the

inspiratory pressure commensurate with the greatest ΔPressure (cmH_2O) / ΔTime (ms).

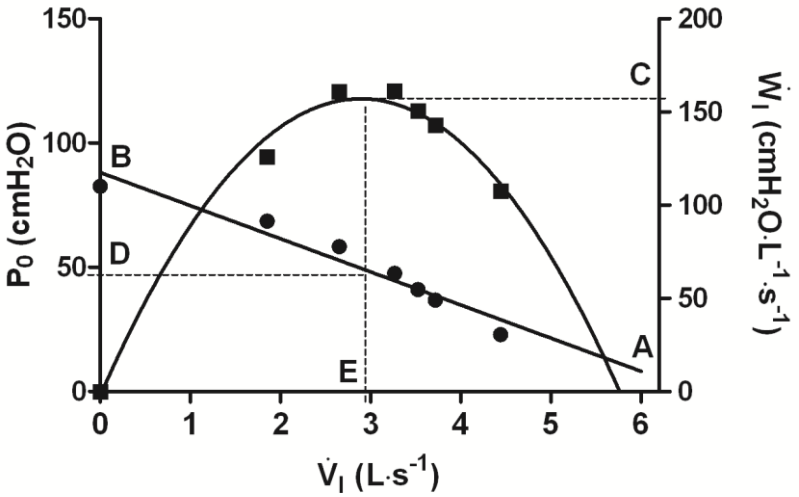


Figure 5.3 Pressure-flow-work calculations for the IMT group at pre-intervention. Values are mean only. SD are removed to improve clarity. A, maximal inspiratory flow at zero pressure ($\dot{V}_{Imax}$); B, maximal inspiratory pressure at zero flow (P_{0max}); C, maximal inspiratory muscle power ($\dot{W}_{Imax}$); D, optimal inspiratory pressure (P_{0opt}); E, optimal inspiratory flow ($\dot{V}_{Iopt}$).

5.1.6 INSPIRATORY MUSCLE ENDURANCE

Inspiratory muscle endurance was assessed using the weighted plunger threshold loading device described in Section 5.1.5 using incremental pressure-threshold loading (ITL). Participants were seated and wore a noseclip. Resistance started at 10 for male or 5 cmH_2O for female participants and was increased by 5 cmH_2O by adding brass weights to the plunger every min until task failure. An audio metronome paced f_B (15 breaths·min^{-1}) and T_I/T_{TOT} (0.5). Participants were instructed to match resting inspiratory flow rates and V_T which were displayed in real-time to the participant and investigator. The breathing pattern was chosen to reflect the same spontaneous breathing pattern adopted during incremental threshold loading using the same device (Johnson et al. 1997). Task failure (endurance time)

was defined as the inability to maintain the correct V_T for three consecutive breaths despite verbal encouragement.

5.1.7 SIX MINUTE WALK TEST

Exercise capacity was assessed using a 6MWT according to published guidelines (ATS 2002), but modified to accommodate space considerations. Participants were instructed to walk as quickly as possible along a 15 m straight, flat, indoor running track and turned around a cone positioned at either end. Immediately after finishing the test measurements were obtained for f_C using short range telemetry (Polar S610; Polar), SpO_2 using infrared fingertip pulse oximetry (Model 8600; Nonin), and RPE for leg discomfort and dyspnoea using Borg's modified CR10 scale (Borg 1982).

5.1.8 ACCELEROMETRY

Accelerometry was measured using the ActiGraph Model GT1M (ActiGraph Manufacturing Technology, Florida, USA) according to current recommendations (Murphy 2009). Participants were instructed to wear the ActiGraph unit on an elastic belt provided by the manufacturer as close to their centre of gravity as possible for 7 days during waking hours and to remove it for sleep and bathing only. Activity was recorded using 5 s epochs and participants completed a log to record whole-body physical activity and when the accelerometer was worn.

The data was downloaded (ActiGraph Lifestyle monitoring System Version 3.3; ActiGraph Manufacturing Technology) and then uploaded to a public domain software package (Mahuffe; http://www.mrc-epid.cam.ac.uk/Research/PA/Downloads.html). Recorded data files were viewed for

signs of malfunction or outliers and checked against participants logs. Based on a similar population (Davis and Fox 2007), data was reduced to provide bands of PAL: sedentary <200; light 200−1999; moderate 2000−3999; vigorous 4000−5274; and very vigorous >5275 count·min^{-1}.

5.1.9 BODY COMPOSITION AND QUESTIONNAIRES

Body composition was measured using bioelectrical impedance (Bodystat 1500; Bodystat, Isle of Man, UK) according to published guidelines (Kyle et al. 2004) to establish the percentage of body fat. PAL and QoL were administered via interview using the Physical Activity Scale for the Elderly (PASE; Washburn et al. 1993) and Older People's Quality of Life Questionnaire (OPQOL-35; Bowling 2009), respectively. Both PASE and OPQOL-35 are scored so that higher scores equal a higher PAL or QoL and scores can range from 0−>400 and 35−175, respectively.

5.1.10 COLLECTION OF BLOOD FOR ASSAYS AND ISOLATION OF PERIPHERAL BLOOD MONONUCLEAR CELLS

Whole blood samples (~10 mL) were taken at rest from an antecubital vein whilst participants were semi-recumbent. Blood was immediately transferred into pre-cooled tubes (SARSTEDT) containing either EDTA for plasma cytokines or lithium heparin for PBMC. Blood for plasma cytokines was immediately centrifuged for 15 min at 1000 x *g* and 5ºC. The plasma supernatant was subsequently removed and stored at -80ºC until further analysis. PBMC were isolated using density gradient centrifugation as stated in Section 2.13.1.

5.1.11 PLASMA CYTOKINE ASSAY

Systemic inflammation was measured with a range of plasma cytokines and concentrations were measured in duplicate using an ultrasensitive electrochemiluminescence multiplex immunoassay which uses a solid-phase microspot assay based on ultra-low-noise charge-coupled device cameras (Meso Scale Discovery, Maryland, USA). To minimise the effect of inter-assay variation, pre- and post-intervention cytokines from both groups were measured during the same assay. The intra-assay (pooled mean of both groups at pre- and post-intervention) and inter-assay CV were 14% and 18%, respectively. The lowest limit of detection (sensitivity) was: IL-1β, 1.44 $pg \cdot mL^{-1}$; interleukin-2, 0.56 $pg \cdot mL^{-1}$; IL-6, 0.19 $pg \cdot mL^{-1}$; interleukin-8, 0.14 $pg \cdot mL^{-1}$; IL-10, 0.89 $pg \cdot mL^{-1}$; interleukin-12 p70, 1.02 $pg \cdot mL^{-1}$; granulocyte macrophage colony-stimulating factor, 0.68 $pg \cdot mL^{-1}$; interferon-γ, 0.86 $pg \cdot mL^{-1}$; and TNF-α, 0.86 $pg \cdot mL^{-1}$. If the lowest limit of detection was not met, participant data (from pre- and post-intervention) was excluded from the analysis. All data for IL-1β was excluded.

5.1.12 MEASUREMENT OF DNA DAMAGE IN PERIPHERAL BLOOD MONONUCLEAR CELLS

Systemic oxidative stress was measured using the alkaline comet assay (Singh et al. 1988) and the modified alkaline comet assay (Collins et al. 1993) to determine levels of DNA damage (DNA single strand breaks and alkali labile lesions) in PBMC as stated in Section 2.13.2.

5.1.13 INSPIRATORY MUSCLE TRAINING AND PLACEBO INTERVENTIONS

The IMT group performed training sessions according to the procedures stated in Section 2.15. The PLA group used a sham hypoxic trainer which was

identical to that used by the IMT group, except that the resistance spring was removed and the lower chamber was loosely packed with aquarium gravel, which was promoted to the participants as being O_2 absorbent, thus reducing the O_2 content of inspired air and mimicking altitude exposure (Johnson et al. 2007; Sonetti et al. 2001). Participants were instructed to breathe normally for 30 consecutive breaths twice daily through the device and to not increase their normal breathing effort. Pulmonary function and maximal mouth pressure were assessed at 2 and 4 weeks during the intervention. During these visits, correct training technique was evaluated in both IMT and PLA groups and the "O_2 absorbent" gravel in the PLA device was also replaced. All participants completed a training diary throughout the study to record adherence to the prescribed intervention and whole-body training sessions. Training sessions for IMT and PLA groups were not performed on the day of laboratory visits.

5.1.14 STATISTICAL ANALYSES

Statistical analyses were performed using SPSS for Windows (IBM). Pulmonary function and MIP / MEP data were analysed using a two-way repeated measures ANOVA to test between-group effects due to 'treatment' (IMT vs. PLA) and within-group effects due to 'intervention' (pre- vs. post-intervention) or 'time' (2, 4 and 8 week). All other data were analysed with independent *t*-tests to test between group effects due to 'treatment' and paired *t*-tests for within group effects due to 'intervention'. Pearson product-moment correlation coefficients were calculated to assess the relationship between selected variables. Reliability was assessed with the CV. Statistical significance was set at $P < 0.05$. Results are presented as mean ± SD.

5.2 RESULTS

One PLA participant withdrew during the intervention due to illness. One IMT participant was removed from the analysis because they completed <70% of the prescribed intervention. Results are therefore presented for 34 participants who completed the study. For the blood analyses, one IMT participant did not consent to blood sampling. For PAL, the accelerometer malfunctioned for one PLA participant during the post-intervention analysis. Therefore, for these variables, results are presented based on 16 participants in each group, respectively.

5.2.1 PRE-INTERVENTION CHARACTERISTICS

There were no significant differences between the groups in any pre-intervention measures apart from $T_{di.FRC}$ which was lower ($P < 0.01$) for the IMT compared to the PLA group. There were no changes in any participant characteristics in both groups post-intervention (Table 5.2). Pre-intervention pulmonary function and maximal mouth pressures were all within normal limits (Table 5.3).

Table 5.2 Participant characteristics for IMT and PLA groups. Values are mean ± SD.

	IMT		PLA	
	Pre-intervention	Post-intervention	Pre-intervention	Post-intervention
Age (years)	69 ± 3		68 ± 3	
Sex (Male/Female)	9/8		11/6	
Height (cm)	167 ± 9	167 ± 10	168 ± 6	168 ± 6
Body mass (kg)	69 ± 12	68 ± 12	72 ± 8	72 ± 8
BMI ($kg \cdot m^2$)	25 ± 3	24 ± 3	26 ± 2	25 ± 3
Body fat (%)	31 ± 7	31 ± 8	31 ± 6	31 ± 7
BP Systolic (mmHg)	135 ± 13	131 ± 13	144 ± 18	140 ± 19
BP Diastolic (mmHg)	81 ± 4	80 ± 4	84 ± 4	80 ± 6

BMI, body mass index; BP, blood pressure.

5.2.2 INTERVENTION

Compliance with the intervention was excellent in both the IMT and PLA groups with 97 ± 5 and 96 ± 7% of the sessions completed resulting in a total of 113 ± 25 and 104 ± 17 individual sessions, respectively. Inspection of training diaries revealed habitual whole-body training remained constant in both groups. Measurements obtained at pre- and post-intervention in both groups are presented in Tables 5.2 to 5.7 and Figure 5.4 and the effects of the intervention are summarised as follows.

5.2.3 PULMONARY FUNCTION AND MAXIMAL MOUTH PRESSURES

Pulmonary function and maximal mouth pressures are shown in Table 5.3. There was a small decrease (3 ± 4%, $P < 0.01$) in FVC in the PLA group, which resulted in a small increase (2 ± 3%, $P < 0.01$) in FEV_1/FVC. PIF increased in both the IMT (35 ± 42%, $P < 0.01$) and PLA (17 ± 29%, $P < 0.05$) groups. The increase from pre-intervention was greater for the IMT compared to the PLA group (time x intervention interaction, $P < 0.05$). MIP was unchanged in the PLA group. In contrast, MIP increased from 82 ± 27 cmH_2O at pre-intervention to 97 ± 23, 100 ± 23, and 103 ± 23 cmH_2O (34 ± 43%, $P< 0.01$) after 2, 4 and 8 weeks of IMT, respectively. No changes were observed in any other pulmonary function measurements or MEP during or post-intervention.

Table 5.3 Pulmonary function and maximal mouth pressures. Values in parentheses represent the percent of predicted values (Enright et al. 1995; Falaschetti et al. 2004; Garcia-Rio et al. 2004). Values are mean ± SD.

	IMT		PLA	
	Pre-intervention	Post-intervention	Pre-intervention	Post-intervention
FVC (L)	3.56 ± 0.82 (107 ± 12)	3.48 ± 0.76 (105 ± 10)	3.67 ± 0.82 (106 ± 12)	3.54 ± 0.63 (102 ± 8)*
FEV_1 (L)	2.63 ± 0.53 (104 ± 11)	2.64 ± 0.53 (104 ± 12)	2.83 ± 0.53 (107 ± 11)	2.79 ± 0.50 (105 ± 11)
FEV_1/FVC (%)	77 ± 3 (98 ± 3)	77 ± 4 (99 ± 5)	78 ± 3 (100 ± 3)	80 ± 5 (102 ± 6)*
PEF ($L \cdot sec^{-1}$)	6.95 ± 1.47 (102 ± 11)	6.90 ± 1.82 (101 ± 16)	7.58 ± 1.47 (105 ± 11)	7.50 ± 1.96 (104 ± 19)
PIF ($L \cdot sec^{-1}$)	4.15 ± 1.26 (100 ± 27)	5.22 ± 1.11 (126 ± 21)*	4.92 ± 1.26 (111 ± 27)	5.52 ± 1.34 (126 ± 22)**
MVV_{10} ($L \cdot min^{-1}$)	103 ± 27	105 ± 30	124 ± 27	121 ± 36
MIP (cmH_2O)	82 ± 27 (97 ± 25)	103 ± 23 (122 ± 20)*	96 ± 27 (107 ± 25)	98 ± 26 (110 ± 23)
MEP (cmH_2O)	124 ± 43 (88 ± 23)	126 ± 38 (90 ± 23)	132 ± 43 (86 ± 23)	128 ± 44 (84 ± 22)

FVC, forced vital capacity; FEV_1, forced expiratory volume in 1 s; PEF, peak expiratory flow; PIF, peak inspiratory flow; MVV_{10}, maximum voluntary ventilation in 10 s; MIP, maximal inspiratory pressure; MEP, maximal expiratory pressure. Significant difference (from pre-intervention $^{*}P < 0.01$, $^{**}P < 0.05$).

5.2.4 DYNAMIC INSPIRATORY MUSCLE FUNCTION AND INSPIRATORY MUSCLE ENDURANCE

Dynamic inspiratory muscle function and inspiratory muscle endurance (ITL) is shown in Table 5.4. P_{0max} increased in both the IMT (27 ± 31%, $P < 0.01$) and PLA (20 ± 45%, $P < 0.05$) groups. No other measurements changed post-intervention in the PLA group. There were increases ($P < 0.05$) in $\dot{W}_{Imax}$ (31 ± 58%) and P_{0opt} (62 ± 151%) and increases ($P < 0.01$) in MRPD (59 ± 84%) and ITL (87 ± 127%) in the IMT group. The increase in $\dot{W}_{Imax}$ was positively correlated with the increase in P_{0opt} (r = 0.96, $P = 0.00$) and ITL time (r = 0.79, $P = 0.00$). The increase in P_{0opt} was positively correlated with the increase in ITL time (r = 0.77, $P = 0.00$).

Table 5.4 Dynamic inspiratory muscle function and inspiratory muscle endurance for IMT and PLA groups. Values are mean ± SD.

	IMT		PLA	
	Pre-intervention	Post-intervention	Pre-intervention	Post-intervention
P_{0max} (cmH_2O)	83 ± 24	100 ± 21*	89 ± 29	97 ± 26**
$\dot{V}_{Imax}$ ($L \cdot s^{-1}$)	7.18 ± 1.98	7.25 ± 1.93	8.30 ± 2.68	7.51 ± 2.37
$\dot{W}_{Imax}$ ($cmH_2O \cdot L^{-1} \cdot s^{-1}$)	171 ± 72	206 ± 70**	216 ± 106	217 ± 104
P_{0opt} (cmH_2O)	48 ± 18	60 ± 11**	56 ± 19	58 ± 16
$\dot{V}_{Iopt}$ ($L \cdot s^{-1}$)	3.25 ± 1.18	3.12 ± 0.75	3.14 ± 0.99	3.15 ± 0.81
P_{0opt} / P_{0max} (%)	58 ± 14	60 ± 5	66 ± 12	60 ± 8
$\dot{V}_{Iopt}$ / $\dot{V}_{Imax}$ (%)	46 ± 12	43 ± 3	39 ± 9	43 ± 6
MRPD ($cmH_2O \cdot ms^{-1}$)	0.35 ± 0.19	0.49 ± 0.19*	0.36 ± 0.15	0.44 ± 0.17
ITL (min)	5.03 ± 2.60	7.50 ± 2.73*	6.93 ± 3.75	7.99 ± 3.52

P_{0max}, maximal inspiratory pressure at zero flow; $\dot{V}_{Imax}$, maximal inspiratory flow at zero pressure; $\dot{W}_{Imax}$, maximal inspiratory muscle power; P_{0opt}, optimal inspiratory pressure; $\dot{V}_{Iopt}$, optimal inspiratory flow; MRPD, maximum rate of inspiratory pressure development; ITL, incremental pressure-threshold loading. Significant difference (from pre-intervention $^{*}P < 0.01$, $^{**}P < 0.05$).

5.2.5 DIAPHRAGM THICKNESS

There were increases ($P < 0.05$) in $T_{di.RV}$ (38 ± 39%) and $T_{di.FRC}$ (28 ± 22%), increase in $T_{di.TLC}$ ($P < 0.01$, 10 ± 17%) and decrease in $T_{di.TR}$ ($P < 0.01$, 17 ± 28%) in the IMT, whereas there were no changes in the PLA group (Table 5.5).

Table 5.5 Diaphragm thickness (T_{di}) measurements for IMT and PLA groups. Values are mean ± SD.

	IMT		PLA	
	Pre-intervention	Post-intervention	Pre-intervention	Post-intervention
$T_{di.RV}$ (mm)	1.60 ± 0.49	2.13 ± 0.50*	1.85 ± 0.53	2.02 ± 0.56
$T_{di.FRC}$ (mm)	1.93 ± 0.50	2.42 ± 0.48*	2.60 ± 0.64†	2.37 ± 0.60
$T_{di.TLC}$ (mm)	3.38 ± 0.57	3.65 ± 0.51**	3.75 ± 0.90	3.58 ± 0.84
$T_{di.TR}$	1.84 ± 0.64	1.41 ± 0.25**	1.86 ± 0.78	1.62 ± 0.53

$T_{di.RV}$, residual volume; $T_{di.FRC}$, functional residual capacity; $T_{di.TLC}$, total lung capacity; $T_{di.TR}$, thickening ratio. Significant difference (from pre-intervention $^{*}P < 0.01$, $^{**}P < 0.05$; between groups $^{\dagger}P < 0.01$).

5.2.6 SIX MINUTE WALK TEST

There were no differences in the 6MWT distance in both groups post-intervention (Table 5.6). No 6MWT measurements changed post-intervention in the IMT group, whereas there was a increase ($P < 0.05$) in f_C (17 ± 27%) and decrease in RPE for leg discomfort (40 ± 61%) in the PLA group. No other measurements changed post-intervention in the PLA group.

Table 5.6 Six minute walk test for IMT and PLA groups. Values are mean ± SD. Percent of predicted values from Troosters et al. (1999).

	IMT		PLA	
	Pre-intervention	Post-intervention	Pre-intervention	Post-intervention
6MWTD (m)	546 ± 85	540 ± 165	554 ± 96	575 ± 97
6MWTD (%Predicted)	125 ± 18	124 ± 35	123 ± 20	128 ± 21
f_C (beats·min^{-1})	98 ± 27	90 ± 29	90 ± 21	103 ± 21**
SpO_2 (%)	97 ± 4	97 ± 2	97 ± 3	98 ± 2
RPE Dyspnoea	1.15 ± 1.32	0.87 ± 1.76	1.43 ± 1.71	0.89 ± 1.26
RPE Leg	0.94 ± 1.54	0.77 ± 1.77	1.38 ± 1.23	0.74 ± 1.30**

6MWTD, six minute walk test distance; f_C, cardiac frequency; SpO_2, estimated arterial oxygen saturation; RPE, rating of perceived exertion. Significant difference (from pre-intervention $^{**}P < 0.05$).

5.2.7 PHYSICAL ACTIVITY LEVELS AND QUALITY OF LIFE

There were no differences in qualitative PAL (assessed by the PASE) or QoL (or in their subcategories) in both groups. For IMT and PLA groups at pre-intervention summed (all subcategories) PAL score was 174 ± 56 and 181 ± 69 and summed QoL score was 134 ± 7 and 137 ± 9, respectively. There were no differences in any quantative PAL (assessed by accelerometry) bands in both groups post-intervention. For IMT and PLA groups pre-intervention MVPA was 46 ± 23 and 37 ± 17 count·min^{-1}.

5.2.8 PLASMA CYTOKINES AND DNA DAMAGE LEVELS IN PERIPHERAL BLOOD MONONUCLEAR CELLS

There were no differences in plasma cytokines (Table 5.7) and DNA damage levels in PBMC (Figure 5.4) in both groups post-intervention.

Table 5.7 Plasma cytokines for IMT and PLA groups. Values in parentheses represent the number of participants included in the analysis. Values are mean ± SD.

	IMT		PLA	
	Pre-intervention	Post-intervention	Pre-intervention	Post-intervention
IL-2 ($pg \cdot mL^{-1}$)	0.50 ± 0.42	0.48 ± 0.41[(9)]	0.88 ± 1.17	0.63 ± 0.55[(6)]
IL-6 ($pg \cdot mL^{-1}$)	1.13 ± 0.38	0.98 ± 0.41[(15)]	2.44 ± 2.81	2.79 ± 4.67[(17)]
IL-8 ($pg \cdot mL^{-1}$)	5.72 ± 4.42	5.15 ± 3.30[(16)]	6.12 ± 5.66	5.42 ± 2.95[(17)]
IL-10 ($pg \cdot mL^{-1}$)	2.11 ± 1.23	1.78 ± 1.06[(12)]	3.40 ± 5.38	2.90 ± 3.18[(16)]
IL-12 p70 ($pg \cdot mL^{-1}$)	1.36 ± 0.58	1.48 ± 0.76[(9)]	2.26 ± 1.63	2.19 ± 1.67[(10)]
GM-CSF ($pg \cdot mL^{-1}$)	2.05 ± 3.24	1.90 ± 2.76[(6)]	1.84 ± 1.54	1.41 ± 1.13[(7)]
IFN-γ ($pg \cdot mL^{-1}$)	2.53 ± 1.70	1.47 ± 1.35[(7)]	2.94 ± 3.36	2.49 ± 2.13[(12)]
TNF-α ($pg \cdot mL^{-1}$)	8.40 ± 3.33	7.78 ± 2.98[(16)]	9.56 ± 6.62	8.79 ± 4.79[(17)]

IL-2, interleukin-2; IL-6, interleukin-6; IL-8, interleukin-8; IL-10, interleukin-10; IL-12 p70, interleukin-12 p70; GM-CSF, granulocyte macrophage colony-stimulating factor; IFN-γ, interferon-γ; TNF-α, tumor necrosis factor-α.

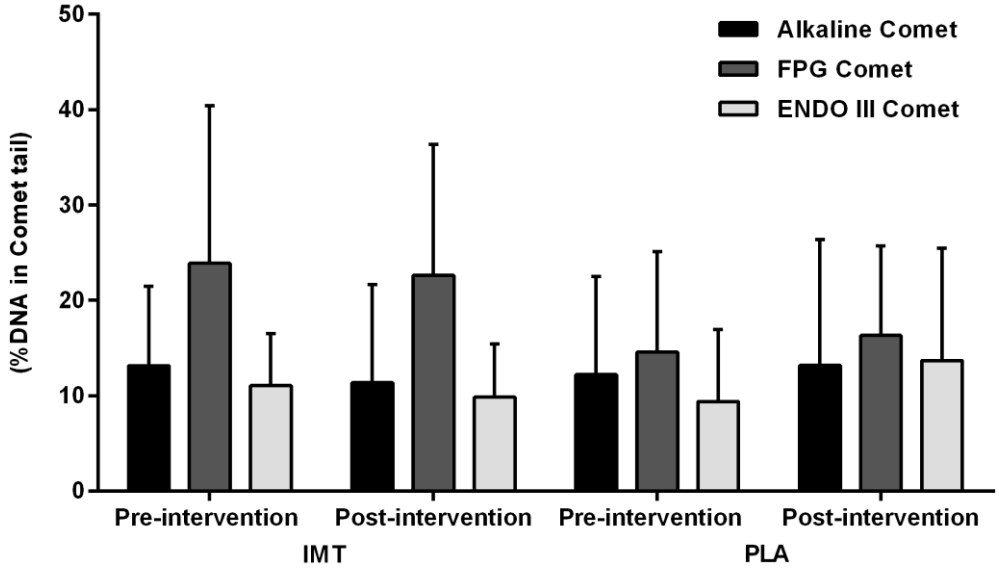


Figure 5.4 DNA damage levels in peripheral blood mononuclear cells for IMT and PLA groups. FPG, formamidopyrimidineglycosylase; ENDO III, endonuclease III for IMT and PLA groups. Values are mean ± SD.

5.3 DISCUSSION

5.3.1 MAIN FINDINGS

The main findings were that IMT increased inspiratory muscle function (strength, endurance and dynamic inspiratory muscle function), structure (T_{di}) and

PIF, but did not change other measures of pulmonary function, resting markers of systemic inflammation (plasma cytokine concentrations) and oxidative stress (DNA damage levels in PBMC), exercise capacity (6MWT distance), quantative and qualitative PAL and QoL. This study is the first to examine the effects of IMT on markers of systemic inflammation and oxidative stress and inspiratory muscle function and structure in older adults. The effects of the intervention are summarised as follows.

5.3.2 PULMONARY FUNCTION AND INSPIRATORY MUSCLE FUNCTION AND STRUCTURE

Apart from PIF, pulmonary function was unchanged in the IMT group. This is in contrast with those who observed an increase in vital capacity after VIH (Belman and Gaesser 1988) and FVC and PEF after IMT/EMT (Watsford and Murphy 2008). In younger adults, the changes following VIH or IMT/EMT in these pulmonary function measures are equivocal (Griffiths and McConnell 2007; Leddy et al. 2007; Verges et al. 2007; Wells et al. 2005). In younger adults, FVC and PEF are never changed following IMT (Brown et al. 2008; Brown et al. 2010; Brown et al. 2012; Johnson et al. 2007). Belman and Gaesser (1988) and Watsford and Murphy (2008) failed to provide a mechanism for the changes in pulmonary function, but it is suggested that this may be a learning effect rather than an adaptation. It is well known that volitional techniques are dependent upon learning and motivation. Therefore, improvements in pulmonary function may be due to participants becoming better at undertaking the forced expiratory manoeuvres that are replicated during training (Polkey and Moxham 2004). This suggestion is supported by the finding in the present study of an increase in PIF and P_{0max} in the PLA group, even with a rigorous familiarisation of the manoeuvres undertaken

before pre-intervention measures. The increase in PIF was statistically greater for the IMT group. It could be suggested that this may be due to an increase in total respiratory system compliance. However, it cannot be ruled out that they also experienced a learning effect from undertaking dynamic inspiratory manoeuvres. Along with the increase in f_C and decrease in RPE for leg discomfort during the 6MWT in the PLA group, this highlights the requirement to employ a legitimate placebo group that will impact participant expectation and motivation.

Inspiratory muscle strength (MIP and P_{0max}) increased in the IMT group by 34 ± 43 and 27 ± 31%, respectively. This is consistent with the increase of 21−39% reported in similar ages (65−71 years) following IMT (Aznar-Lain et al. 2007; Huang et al. 2011; Watsford and Murphy 2008). It is interesting to note that a rapid increase in MIP was observed from 82 ± 27 to 97 ± 23 cmH_2O (+23 ± 28%) within just 2 weeks of IMT. This has been reported previously in younger participants with 14% and 28% increases in MIP observed after 1 and 2 weeks of IMT, respectively (Downey et al. 2007; Huang et al. 2003). Such rapid training-induced increases in limb muscle strength are usually ascribed to neural adaptations, with structural alterations becoming evident after about 6−8 weeks of training (Kraemer et al. 1996). However, the signalling pathways that elicit structural alterations in inspiratory muscles might arise earlier than anticipated because of the greater training frequency with IMT (up to 14 sessions per wk^{-1}) (Kraemer et al. 1996). In support, diaphragm thickness and type II fibre size in the external intercostals increased by 8−12 and 21%, respectively, following 4−5 weeks of IMT (Downey et al. 2007; Enright et al. 2006; Ramirez-Sarmiento et al. 2002). These diaphragm thickness findings were confirmed in the present study, but at all lung volumes. The stimuli for diaphragm hypertrophy with IMT are poorly understood but recently it has been shown that

growth hormone and cortisol are increased in young and healthy males immediately after a single 15 min VIH training session, performed 12 days after of similar training (Sartorio et al. 2012).

An increase in the maximal power of the inspiratory muscles ($\dot{W}_{Imax}$) was observed in the IMT group which was solely due to an increase in P_{0max} and not maximal inspiratory flow rate ($\dot{V}_{Imax}$). It may seem contradictory that a change in $\dot{V}_{Imax}$ was not observed but PIF increased (as they represent the same manoeuvre). This, however, can be explained by the type of measurement and analysis performed. PIF is calculated from the inspiratory limb of a flow-volume loop and represents unloaded shortening of the inspiratory muscles whereas $\dot{V}_{Imax}$ is calculated from extrapolation of a linear least squares representation of the pressure-flow data undertaken at six discrete resistances. Thus, the linear regression of $\dot{V}_{Imax}$ is dependent upon the inspiratory muscles contracting under loading. The increase in $\dot{W}_{Imax}$ is consistent with other reports in younger adults, but unlike the present finding, this is with a combination of an increase in P_{0max}, and to a lesser extent an increase in $\dot{V}_{Imax}$ (Romer et al. 2002a; Romer and McConnell 2003). This finding suggests that in healthy older adults and with an initial training load of 50% MIP, P_{0max} is only responsive to the training stimuli. This finding is supported by the observation that 5 weeks of IMT (with an initial training load of 40−50% MIP) decreased fast twitch (type II) fibres by 15% in the external intercostals of 65 year old COPD patients (Ramirez-Sarmiento et al. 2002). These authors' also observed a 50% increase in inspiratory muscle endurance with the shift in fibre type. There was also an increase in inspiratory muscle endurance (ITL) in the IMT group which is consistent with Watsford and Murphy (2008). Several mechanisms, including the adaptations to inspiratory muscle morphology, may explain this finding. The increase

in optimal inspiratory pressure (P_{0opt}) as a percentage of P_{0max} (P_{0opt} / P_{0max}) would result in inspiratory muscle contractions being performed at a lower proportion of the maximum capacity at a given workload during ITL. This may reduce motor-unit recruitment and delay the onset of fatigue, thus enhancing endurance (McConnell and Romer 2004). There may also be a decrease in the O_2 cost of breathing during ITL, which would improve inspiratory muscle efficiency at a given load (Turner et al. 2012).

The increase in MRPD in the IMT group is consistent with the finding in younger adults (Romer et al. 2002a; Romer and McConnell 2003). The precise mechanism(s) for these increases are unknown, but may be explained by possible resistance training-induced adaptations to the firing frequency of motor units (Kamen 2005). This suggestion is supported by the observation that motor unit firing frequency is higher in the rectus femoris of strength trained older adults than age-matched untrained controls (Leong et al. 1999). Furthermore, motor unit firing frequency is increased in the vastus lateralis of untrained older adults following six weeks of lower-body resistance training (Kamen and Knight 2004). The functional benefits of the increase in MRPD are unclear, but it has been suggested that it would allow for a quicker inspiratory muscle contraction time and allow expiration to be prolonged, which may maintain end-expiratory lung volume, allowing the diaphragm to contract nearer its optimal length-tension relationship, and reduce the elastic recoil forces to be overcome (Romer et al. 2002a).

5.3.3 EXERCISE CAPACITY, PHYSICAL ACTIVITY LEVELS AND QUALITY OF LIFE MEASURES

There were no changes in exercise capacity (6MWT distance), qualitative and quantative PAL or QoL in the IMT group. The exercise capacity findings are in

contrast with others who have reported improvements in 6MWT distance (Huang et al. 2011) and endurance time during treadmill walking at the first ventilatory threshold following IMT (Aznar-Lain et al. 2007). The differences in the findings may be explained by baseline exercise training status. Huang et al. (2011) reported that 6MWT distance in participants at pre-intervention were 90 ± 16% of predicted values (Troosters et al. 1999), suggesting that the cardiorespiratory response to the 6MWT may have been limited. In the present study 6MWT distance in participants at pre-intervention was 123−125% of predicted values (Troosters et al. 1999). If a functional consequence of IMT is to improve 6MWT distance in older adults then it may be those with a low baseline status who show the greatest improvement (Bouchard and Rankinen 2001). Alternatively, Huang et al. (2011) reported that they used American Thoracic Society guidelines for the 6MWT (ATS 2002). These guidelines do not, however, recommend a familiarisation session (Brooks et al. 2003) and Huang et al. (2011) did not report that one was undertaken. In older adults, at least two attempts of the 6MWT are required to obtain reliable measurements (Kervio et al. 2003) and this was undertaken in the present study.

This is the first study to report qualitative PAL, but quantative PAL using accelerometry has demonstrated that MVPA is increased in six older adults following IMT (Aznar-Lain et al. 2007). Aznar-Lain et al. (2007) suggested this was due to an increase in the intensity of exercise performed, as no other accelerometry measure changed following IMT. The low sample size of this study makes the finding difficult to interpret.

The QoL results disagree with Huang et al. (2011) who observed an increase in the physical subcategory of the SF-36 QoL questionnaire. Differences in the type of questionnaire used could explain the findings (SF-36 vs. OPQOL-35), or

alternatively, it could be suggested that baseline economical or health (mental and physical) status of participants could account for the differences. The SF-36 however is a generic measure as opposed to one that specifically examines the older adult like the OPQOL-35.

5.3.4 PLASMA CYTOKINES AND DNA DAMAGE LEVELS TO PERIPHERAL BLOOD MONONUCLEAR CELLS

It was hypothesised that IMT may attenuate markers of systemic inflammation and/or oxidative stress in older adults. Systemic low-level inflammation can be defined as a 2−4 fold increase in the resting concentration of inflammatory mediators (Bruunsgaard 2006). Although it is unreliable to compare cytokine concentrations from different assays, plasma IL-6 concentrations at rest in Chapters 3 and 4 were 0.5−0.7 $pg \cdot mL^{-1}$. In the present study, pre-intervention plasma IL-6 concentrations were 1.13 and 2.44 $pg \cdot mL^{-1}$ for IMT and PLA groups, respectively. Therefore, the present findings suggest that systemic inflammation was evident in older adults. Furthermore, the plasma TNF-α concentrations reported in the present study compare well to Bruunsgaard (2006) (Figure 1.33). The present study also reported a large variance in plasma cytokines. This may relate to, in part, a lack of sensitivity in the assay used, and small differences in the age or health status of study participants (Bruunsgaard 2006).

Whilst IMT did not change the resting plasma concentration of nine cytokines and DNA damage levels in PBMC, it cannot be excluded whether IMT attenuated local respiratory muscle cytokines and/or oxidative stress. A diaphragmatic or intercostal biopsy would allow this area to be explored. However, this is a very invasive measurement that requires open surgery or thoracscopy under general anaesthesia and thus for these healthy individuals this would not be ethical. It could

be that the improvement in inspiratory muscle structure and function did not reach a threshold necessary to achieve a potential systemic reduction in cytokines and/or DNA damage levels in PBMC. Previous studies have shown that whole-body resistance or endurance training can decrease resting systemic cytokine concentrations (Kohut et al. 2006; Nicklas et al. 2008; Phillips et al. 2010; Prestes et al. 2009) and markers of oxidative stress (Fatouros et al. 2004; Parise et al. 2005; Takahashi et al. 2012). However, the respiratory muscles only weigh ~960 g (Freedman et al. 1983) and represent ~3% of total body mass (Robertson et al. 1977). Thus, IMT only targets a small muscle group and although they do contribute to systemic cytokines concentrations through oxidative stress during increased respiratory muscle work, IMT does not attenuate this under resting conditions.

5.4 CONCLUSIONS

RMT has been promoted as a tool to attenuate the age-related decline in inspiratory muscle function and can "increase resistance to fatigue", "improve respiratory muscle efficiency" and "alleviate dyspnoea" all resulting in "improved exercise performance". It has been demonstrated that using a rigorous, placebo controlled design with an adequate sample size, IMT increases inspiratory muscle function (strength, endurance, dynamic inspiratory muscle function), structure (T_{di}) and PIF, but does not change other measures of pulmonary function, resting markers of systemic inflammation (plasma cytokine concentrations) and oxidative stress (DNA damage levels in PBMC), exercise capacity (6MWT distance), quantative and qualitative PAL and QoL in older adults. Therefore, for healthy older adults without respiratory or cardiovascular limitations and with IMT as an RMT device, the present study supports the manufacturers' claims that IMT can "increase resistance to

fatigue", but not that RMT can "alleviate dyspnoea" or "improve exercise performance".

CHAPTER 6 – INSPIRATORY MUSCLE TRAINING DOES NOT CHANGE LACTATE MINIMUM CYCLING POWER OR RESPIRATORY MUSCLE RECRUITMENT PATTERNS DURING THE TEST

6.0 INTRODUCTION

In Chapter 3 the lactate minimum test was used to estimate MLSS intensity. The MLSS represents the highest exercise intensity at which $[La^-]_B$ remains stable over time and is widely acknowledged as an important determinant of endurance exercise performance (Johnson et al. 2009; Johnson and Sharpe 2011; Jones and Doust 1998; MacIntosh et al. 2002). Unfortunately, direct MLSS estimation is laborious, requiring multiple constant intensity exercise tests (typically lasting 30 min) over a range of exercise intensities. The lactate minimum test was originally developed by Tegtbur et al. (1993) to estimate MLSS running velocity. It has since been adapted for MLSS prediction in cycling with recent evidence suggesting a good agreement between the intensities of the lactate minimum test and MLSS (Johnson et al. 2009; Johnson and Sharpe 2011; Knoepfli-Lenzin and Boutellier 2011; MacIntosh et al. 2002; Simoes et al. 2009). The test comprises three consecutive exercise phases: 1) a lactate elevation phase; 2) a recovery phase; and 3) an incremental phase in which with increasing intensity $[La^-]_B$ decreases (net lactate clearance) to a nadir (the lactate minimum) and then increases (net lactate appearance). The $[La^-]_B$ is determined by rates of lactate release into the interstitium of circulation and consumption by adjacent or remote consuming oxidative muscle fibres or organs (the lactate shuttle; Brooks 2000).

Two recent systematic reviews with meta-analyses have reported that RMT can improve endurance exercise performance (Hajghanbari et al. 2012; Illi et al. 2012). While the mechanisms underlying such performance improvements remain speculative, a common observation after RMT is a reduction in $[La^-]_B$ during maximal incremental cycling (Spengler et al. 1999), constant power exercise until the limit of tolerance (Bailey et al. 2010; Boutellier and Piwko 1992; Leddy et al. 2007;

Spengler et al. 1999), and exercise at MLSS (Chapter 3; McConnell and Sharpe 2005). The mechanism(s) by which RMT reduces $[La^-]_B$ remains equivocal, but may be due to either increased lactate uptake and metabolism or decreased efflux by the trained respiratory muscles (Spengler et al. 1999). While it is known that IMT does not change the cycling power at which MLSS corresponds to (McConnell and Sharpe 2005) it is unknown whether it changes MLSS prediction from the lactate minimum test.

During heavy constant load (Babcock et al. 1995; Babcock et al. 1995; Babcock et al. 1996; Babcock et al. 1998; Babcock et al. 2002; Johnson et al. 1993; Mador et al. 1993) and maximal incremental (Romer et al. 2007) exercise until the limit of tolerance there is a progressive increase in the contribution from the less efficient accessory inspiratory (and expiratory) muscles and a decrease in the contribution from the diaphragm to the ventilatory response. Less efficient accessory muscles may contribute more to the ventilatory response because the diaphragm is beginning to accumulate metabolites or fatigue which reflexly inhibits further diaphragm recruitment and triggers the recruitment of additional inspiratory (and expiratory) muscles (Jammes et al. 1986). It has been hypothesised that RMT may alter the breathing pattern and/or delay recruitment from fatiguing accessory muscles during exercise because of an increase in fatigue resistance and/or a desensitising of sensory afferents within the respiratory muscles (McConnell and Romer 2004). This hypothesis, however, remains untested.

Therefore, the aim of this study was to examine the effects of IMT on an estimation of MLSS using the lactate minimum test, and to examine if the contribution of the respiratory muscles to the ventilatory response would change during the test. It was hypothesised that following IMT there would be a decrease in

$[La^-]_B$ during the lactate minimum test which would change the estimated MLSS intensity and that there would be a delay in the recruitment of accessory respiratory muscles.

6.1 METHODS

6.1.1 PARTICIPANTS

Twelve non-smoking recreationally active young males provided written, informed consent to participate in the study, which was approved by the Nottingham Trent University ethics committee (Appendix 1 and 2). Participants followed the pre-experimental instructions outlined in Section 2.0.

6.1.2 EXPERIMENTAL DESIGN

Participants attended the laboratory on two separate occasions, each separated by 48 h and both took place at the same time of day, before and after a 6 week intervention where participants were randomly, and equally, divided into an IMT or PLA group. During the first and third visits, participants were familiarised or re-familiarised with all testing procedures and pulmonary function and MIP were measured. During the second and fourth visits, participants performed a lactate minimum cycling test to estimate MLSS power.

6.1.3 PULMONARY FUNCTION AND MAXIMAL INSPIRATORY MOUTH PRESSURE

Pulmonary function and MIP were assessed according to the procedures stated in Sections 2.3 and 2.4, respectively. MIP was measured at 2 and 4 weeks during the intervention in both IMT and PLA groups.

6.1.4 LACTATE MINIMUM TEST

The lactate minimum test was performed following the procedures of Johnson et al. (2009) as stated in Section 2.16. Each test was preceded by a 5 min rest period and comprised three consecutive phases: (i) lactate elevation phase (LEP) comprising maximal incremental exercise; (ii) 8 min recovery phase at 60 W (REC); and (iii) incremental phase (INC) comprising five consecutive 4 min stages at intensities of 45, 50, 55, 60, and 65% of the $\dot{W}_{max}$ achieved during the LEP. During the LEP power output was increased by 10 W every 15 s so that exercise intolerance (<60 revs·min^{-1}) occurred in approximately 10 min. The final power defined $\dot{W}_{max}$ and the highest oxygen uptake recorded over any 30 s period defined $\dot{V}O_{2peak}$. During the final seconds of each stage of the INC arterialised venous blood was sampled from a dorsal hand vein via an indwelling 21-G cannula (McLoughlin et al. 1992) for the measurement of $[La^-]_B$, which was determined using an automated analyser (Biosen C_line Sport; EKF Diagnostics). Arterialisation was ensured by immersing the hand in water at 40ºC for 10 min prior to cannulation and by warming the hand during trials using an infrared lamp. The lactate minimum power (i.e. estimated MLSS power) was calculated as the nadir of a 2nd order polynomial fitting $[La^-]_B$ against power of each increment. Measurements were taken for f_C using short range telemetry (Polar S610; Polar) and SpO_2 using infrared fingertip pulse oximetry (Model 8600; Nonin) at rest and at termination of the LEP (MAX).

6.1.5 FLOW, PULMONARY GAS EXCHANGE AND PRESSURE MEASUREMENTS

During all trials participants wore a facemask (model 7940; Hans Rudolph) connected to a pneumotachograph and ventilatory and pulmonary gas exchange responses were measured breath by breath (ZAN 600USB; Nspire Health). A two-

way non-rebreathing valve (model 2730; Hans Rudolph) was attached distally to the pneumotachograph and on the expiratory port a Fleisch no. 3 pneumotachograph was attached and connected to a differential pressure transducer (± 2.5 cmH_2O) (TSD160A; BIOPAC Systems) and differential bridge amplifier (DA100C; BIOPAC Systems) to allow alignment of flow and pressure signals. P_e and P_g were measured according to the procedures stated in Section 2.6. P_{di} was calculated by subtracting P_e from P_g. P_{di} and P_e were integrated over the period of inspiratory flow and multiplied by f_B and labelled PTP_{di} and PTP_e, respectively. Non-physiological flows and pressures that resulted from swallowing, coughing and breath holding, were identified by visual inspection and removed.

6.1.6 INSPIRATORY MUSCLE TRAINING AND PLACEBO INTERVENTIONS

The IMT group performed training sessions according to the procedures stated in Section 2.15. The PLA group inhaled one puff twice daily for 6 weeks from a sham metered dose inhaler (Vitalograph). The inhaler contained compressed air only but was promoted to the participants as delivering a novel drug that reduces breathing-induced inflammation. During the post-intervention period the IMT and PLA group reduced their training frequency to 2 $d \cdot wk^{-1}$. These maintenance sessions were performed 48 h before and 48 h after experimental trials. This has been shown to maintain improvements in inspiratory muscle function post-IMT (Romer and McConnell 2003). All participants completed a training diary throughout the study to record adherence to the prescribed intervention and whole-body training sessions.

6.1.7 STATISTICAL ANALYSES

Data collected were aligned to fixed percentages of $\dot{W}_{max}$ during LEP and ensemble averaged for the final minute of REC and the final 30 s of INC. Statistical analyses were performed using SPSS for Windows (IBM). Groups were compared for pre-intervention characteristics using independent *t*-tests. A two-way repeated measures ANOVA were used to test between-group effects due to 'treatment' (IMT vs. PLA) and within-group effects due to 'intervention' (pre- vs. post-'treatment') or 'time' (10, 20, 30, 40, 50, 60, 70, 80, 90 and 100% $\dot{W}_{max}$ of LEP, REC and 45, 50, 55, 60 and 65% $\dot{W}_{max}$ of INC). Planned pairwise comparisons were made with repeated measures *t*-tests and with Bonferroni adjustment. Statistical significance was set at $P < 0.05$. Results are presented as mean ± SD.

6.2 RESULTS

Age, body mass, height, pulmonary function and $\dot{V}O_{2peak}$ were not different between IMT and PLA groups (Table 6.1). $\dot{W}_{max}$ and lactate minimum power were higher ($P < 0.01$) for the IMT compared to the PLA group (Table 6.1). For all other pre-intervention measurements there were no differences between the groups. Compliance with the intervention was excellent in both the IMT and PLA groups with 96 ± 1 and 93 ± 1% of sessions completed, respectively. Pre-intervention pulmonary function and MIP were all within normal limits (Table 6.1). MIP was unchanged post-intervention in the PLA group. In contrast, MIP was 168 ± 49 cmH_2O at pre-intervention and increased to 183 ± 41, 191 ± 37, and 201 ± 39 cmH_2O (+24%) ($P < 0.01$) after 2, 4 and 6 wk of IMT, respectively. Pulmonary function, $\dot{V}O_{2peak}$ and $\dot{W}_{max}$ were unchanged in both groups post-intervention.

Table 6.1 Pre-intervention descriptive characteristics, pulmonary function and maximal inspiratory pressure for IMT and PLA groups. Values in parentheses are the percent of predicted values (Quanjer et al. 1993; Wilson et al. 1984). Values are mean ± SD.

	IMT	PLA
Age (yr)	27 ± 7	22 ± 3
Body mass (kg)	80 ± 6	74 ± 7
Height (cm)	181 ± 5	177 ± 4
FVC (L)	5.58 ± 0.44 (104 ± 7)	5.61 ± 0.67 (105 ± 9)
FEV_1 (L)	4.49 ± 0.46 (100 ± 8)	4.53 ± 0.51 (100 ± 10)
FEV_1/FVC (%)	81 ± 6 (99 ± 6)	81 ± 6 (98 ± 7)
MVV_{10} ($L \cdot min^{-1}$)	210 ± 2 (111 ± 14)	192 ± 33 (102 ± 19)
MIP (cmH_2O)	168 ± 49 (157 ± 9)	136 ± 32 (113 ± 26)
$\dot{V}O_{2peak}$ ($mL \cdot kg^{-1} \cdot min^{-1}$)	51.7 ± 6.2	47.0 ± 5.7
$\dot{W}_{max}$ (W)	378 ± 22*	342 ± 21
Lactate minimum power (W)	199 ± 8*	170 ± 19

FVC, forced vital capacity; FEV_1, forced expiratory volume in 1 s; MVV_{10}, maximum voluntary ventilation in 10 s; MIP, maximal inspiratory pressure; $\dot{V}O_{2peak}$, peak oxygen uptake; $\dot{W}_{max}$, maximum power output. Significant difference (between groups * $P < 0.01$).

6.2.1 PHYSIOLOGICAL RESPONSES TO THE LACTATE MINIMUM TEST

The $[La^-]_B$ response to the lactate minimum test (Figure 6.1) and the lactate minimum power (Table 6.1) was unchanged post-intervention for both groups. Apart from PTP_{di}/PTP_e, breathing and respiratory muscle recruitment pattern and pulmonary gas exchange variables all increased during the LEP, followed by a rapid decrease during REC and a progressive increase during INC (Figures 6.2 and 6.3 and Table 6.2). At the start of the test, PTP_{di}/PTP_e gradually increased, but then progressively decreased through the duration of the test indicating a greater contribution from the accessory respiratory muscles, and a lesser contribution from the diaphragm to $\dot{V}_E$.

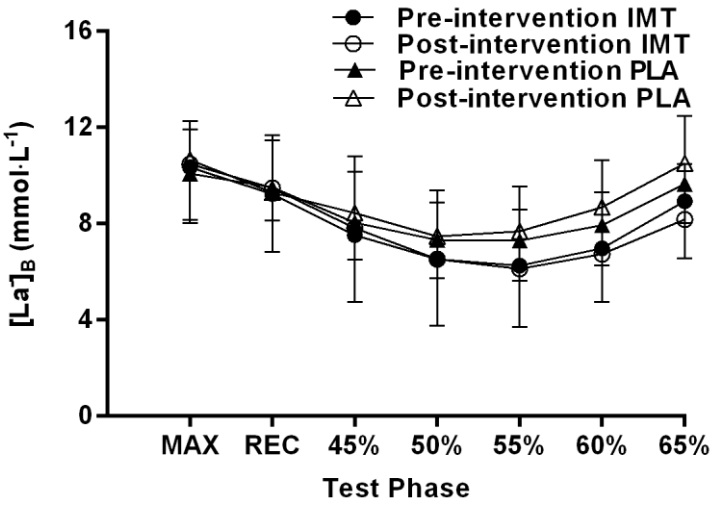


Figure 6.1 Blood lactate concentration ($[La^-]_B$) at the termination of the lactate elevation phase (MAX), during the recovery phase (REC) and at 45, 50, 55, 60 and 65% of the incremental phase for IMT and PLA groups.

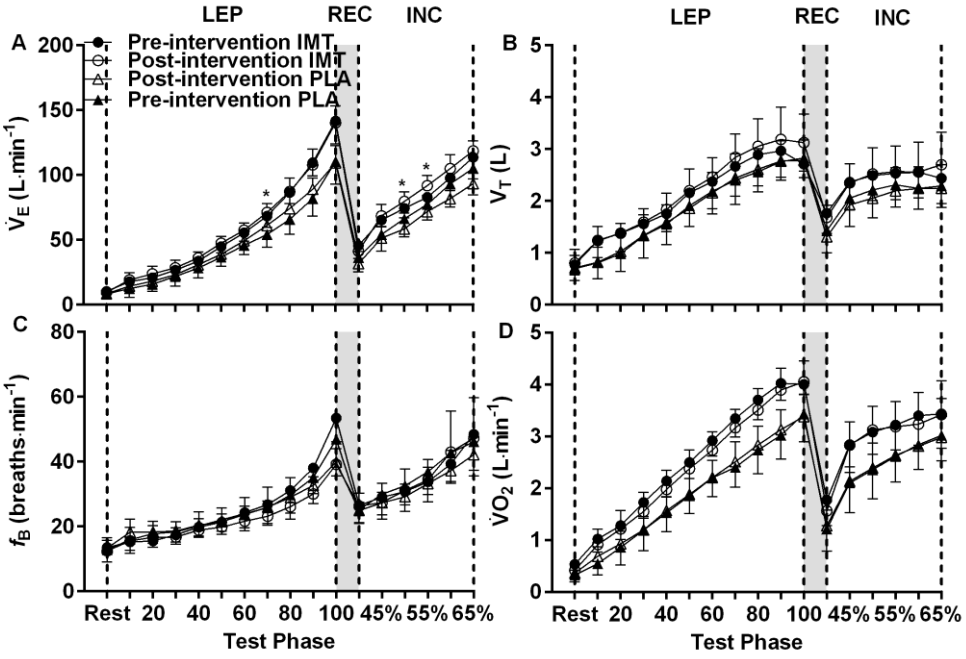


Figure 6.2 Minute ventilation ($\dot{V}_E$; A), tidal volume (V_T; B), breathing frequency (f_B; C) and oxygen uptake ($\dot{V}O_2$; D) for IMT and PLA groups. Dashed lines represent the start and finish of the lactate elevation phase (LEP), recovery phase (shaded area; REC) and incremental phase (INC). Significant difference (from pre-intervention * $P < 0.05$) for the IMT group.

Table 6.2 Pre-intervention physiological responses to the lactate minimum test at termination of the lactate elevation phase (MAX), during the recovery phase (REC) and at 65% of maximum power output during the incremental phase (INC) for IMT and PLA groups.

	MAX	REC	65% of INC
Pre-intervention IMT			
$\dot{V}CO_2$ ($L \cdot min^{-1}$)	5.5 ± 0.5	1.7 ± 0.5	4.1 ± 0.5
RER	1.4 ± 0.1	1.0 ± 0.2	1.2 ± 0.2
P_{ETO2} (mmHg)	120 ± 3	107 ± 7	116 ± 3
P_{ETCO2} (mmHg)	41 ± 4	39 ± 3	40 ± 2
T_I/T_{TOT}	0.50 ± 0.01	0.47 ± 0.02	0.49 ± 0.01
Pre-intervention PLA			
$\dot{V}CO_2$ ($L \cdot min^{-1}$)	4.3 ± 0.5	1.2 ± 0.4	3.4 ± 0.7
RER	1.3 ± 0.0	1.0 ± 0.2	1.1 ± 0.1
P_{ETO2} (mmHg)	115 ± 6	106 ± 8	116 ± 5
P_{ETCO2} (mmHg)	41 ± 4	38 ± 2	39 ± 3
T_I/T_{TOT}	0.50 ± 0.01	0.46 ± 0.02	0.50 ± 0.02

$\dot{V}CO_2$, carbon dioxide production; RER, respiratory exchange ratio; P_{ETO2}, partial pressure of end tidal oxygen; P_{ETCO2}, partial pressure of end tidal carbon dioxide; T_I/T_{TOT}, duty cycle.

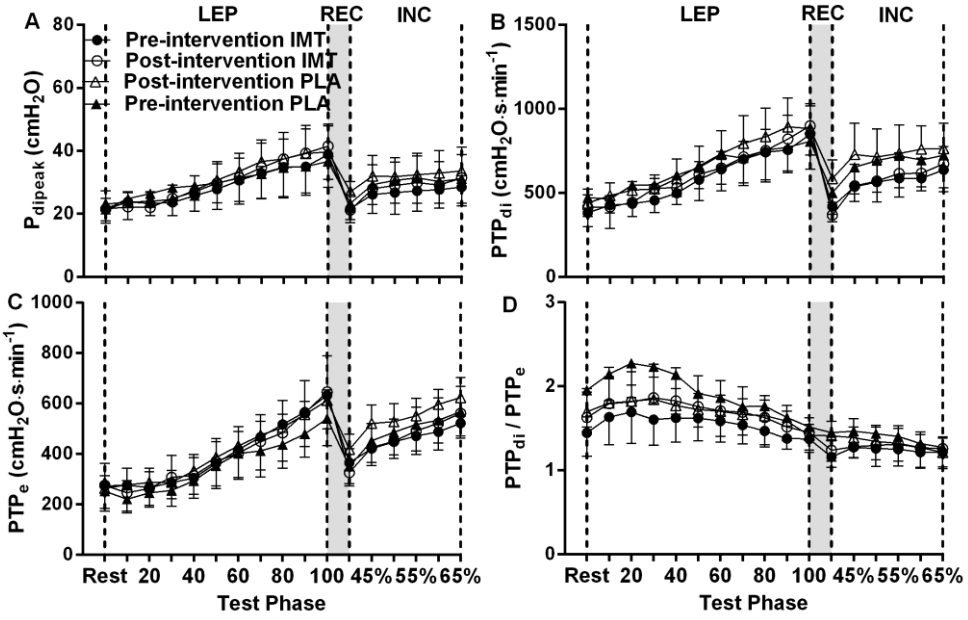


Figure 6.3 Peak transdiaphragmatic pressure (P_{dipeak}; A), diaphragm pressure-time product (PTP_{di}; B), inspiratory muscle pressure-time product (PTP_e; C) and the relative contribution of diaphragm to the inspiratory muscle pressure-time product (PTP_{di}/PTP_e; D) for IMT and PLA groups. Dashed lines represent the start and finish of the lactate elevation phase (LEP), recovery phase (shaded area; REC) and incremental phase (INC).

6.2.2 EFFECTS OF THE INTERVENTION

$\dot{V}_E$ was increased in the IMT group (main effect for intervention, $P < 0.05$), which was due to a strong trend for an increased V_T (main effect for intervention, $P = 0.051$) (Figure 6.2). No other differences in breathing and respiratory muscle recruitment pattern or pulmonary gas exchange variables were observed post-intervention in either group (Figures 6.2 and 6.3 and Table 6.2). For both IMT and PLA groups f_C (174 ± 13 vs. 178 ± 10 and 167 ± 12 vs. 170 ± 10 beats·min^{-1}) and SpO_2 (98 ± 2 vs. 96 ± 3 and 97 ± 3 vs. 96 ± 2%) at MAX were not different post-intervention.

6.3 DISCUSSION

6.3.1 MAIN FINDINGS

The main findings are that in contrast to the hypothesis and following IMT there was no change to the estimation of MLSS using the lactate minimum test and there were no differences in the contribution of the respiratory muscles to the ventilatory response during the test.

The lactate minimum test differs from other exercise protocols used to evaluate the effects of RMT and therefore it is difficult to compare the present findings. The lactate minimum test involves an incremental test performed until exhaustion (LEP), a short active recovery phase (REC) where the ventilatory response does not decrease to resting levels, and a sub-maximal incremental phase (INC) where ventilation progressively increases, but only reaches near-maximal levels in the final stage.

6.3.2 EFFECTS OF IMT ON $[La^-]_B$ AND MLSS ESTIMATION

There was no effect of IMT on $[La^-]_B$ during the lactate minimum test. During (or immediately following) maximal incremental exercise until the limit of volitional tolerance, $[La^-]_B$ has been observed to either decrease (Spengler et al. 1999; Volianitis et al. 2001), decrease but not significantly (Boutellier et al. 1992; Romer et al. 2002a) or remain unchanged (McMahon et al. 2002). The differences cannot be explained by study protocol or training status as McMahon et al. (2002) and Spengler et al. (1999) utilised the same incremental protocol and participants that were endurance trained ($\dot{V}O_{2peak}$ > 60 mL·kg·min^{-1}). The data from Volianitis et al. (2001) is questionable as following the intervention period the placebo group also demonstrated a similar reduction in $[La^-]_B$ during exercise.

$[La^-]_B$ reflects both the rate of lactate release into, and removal from, the systemic circulation (Gladden 2004). Throughout the majority of maximal incremental exercise, net lactate production is probably negligible, as there is there is sufficient capacity for tissues (e.g., active skeletal muscle, the kidney and the liver (Brooks 2000)) to clear the lactate produced. Thus, any potential RMT-mediated increases in oxidative (Ramirez-Sarmiento et al. 2002) and/or lactate transport capacity of the inspiratory muscles (Brown et al. 2008; Brown et al. 2010; Brown et al. 2012) may not be evident on $[La^-]_B$ until there is an increase in net lactate production. This probably occurs at higher workloads, where $\dot{V}_E$ is relatively high compared to MVV, and the respiratory muscles engage in net lactate production (Boutellier et al. 1992; Romer et al. 2002a; Spengler et al. 1999; Spengler et al. 2000). This relatively small change in $[La^-]_B$ may only by evident with larger (n = 20 participants) sample sizes (Spengler et al. 1999). However, in the presnt study equivocal findings were observed for $[La^-]_B$ at MAX, with both increases and

decreases following IMT. In contrast, most studies report a decrease in $[La^-]_B$ during constant load exercise to the limit of tolerance (Bailey et al. 2010; Boutellier et al. 1992; Leddy et al. 2007; Romer et al. 2002a; Spengler et al. 1999) or at MLSS (Chapter 3; McConnell and Sharpe 2005), where throughout the majority of exercise there is limited capacity to match further lactate appearance with an equal rate of lactate clearance. In this situation, RMT-mediated respiratory muscle adaptations may have the capacity to augment $[La^-]_B$.

It was postulated that IMT may affect $[La^-]_B$ and therefore change the MLSS estimation. This was based on evidence that IMT may reduce $[La^-]_B$ and have less probable effects on $\dot{W}_{max}$. Most well controlled studies have found no effect of RMT on $\dot{W}_{max}$ during maximal incremental exercise to the limit of volitional tolerance (Hajghanbari et al. 2012; Illi et al. 2012). Romer et al. (2002a) have observed that when the inspiratory muscles are unloaded using a PAV, there is no increase in $\dot{W}_{max}$, even with significant reductions in whole-body $\dot{V}O_2$ and perceptual (leg discomfort and dyspnoea) responses. Moreover, without inspiratory muscle unloading, maximal incremental exercise did not elicit substantial diaphragm fatigue. Taken together these findings suggest that neither inspiratory muscle work, nor diaphragm fatigue, limit maximal incremental exercise performance, two of the mechanisms by which RMT may exert an ergogenic effect upon performance (Table 1.1).

6.3.3 Effects of IMT on the contribution of the respiratory muscles to ventilation

During the lactate minimum test, PTP_{di}/PTP_e gradually increased but progressively decreased through the duration of the test, indicating a greater contribution from the accessory inspiratory muscles, and a lesser contribution from the diaphragm to $\dot{V}_E$. This has previously been observed during heavy constant load

exercise (Babcock et al. 1995; Babcock et al. 1995; Babcock et al. 1996; Babcock et al. 1998; Johnson et al. 1993; Mador et al. 1993) and to a lesser extent, maximal incremental exercise (Romer et al. 2007). Less efficient accessory muscles may contribute more to the ventilatory response because the diaphragm is beginning to accumulate metabolites or fatigue, which reflexly inhibits further diaphragm recruitment and triggers the recruitment of additional inspiratory (and expiratory) muscles (Jammes et al. 1986). It was hypothesised that IMT may alter the breathing pattern and/or delay recruitment from fatiguing accessory muscles during the lactate minimum test, because of an increase in respiratory muscle fatigue resistance and/or a desensitising of sensory afferents within the respiratory muscles (McConnell and Romer 2004). However, no change in respiratory muscle recruitment patterns following IMT was found. Why IMT did not alter this response may be due to the type of exercise performed. Indeed, when the inspiratory muscles are unloaded with a PAV, PTP_{di}/PTP_e remains at resting values during heavy constant load exercise (Babcock et al. 2002), but PTP_{di}/PTP_e still increases during maximal incremental exercise (Romer et al. 2007). These findings suggest that reducing the inspiratory WOB during maximal incremental exercise does not alter respiratory muscle recruitment patterns. It may be that during the lactate minimum test, there is an insufficient accumulation of metabolic stimuli and/or diaphragmatic fatigue to make any RMT-mediated adaptations evident.

6.4 CONCLUSION

In conclusion, IMT does not change the estimation of MLSS using the lactate minimum test and it does not change the contribution of the respiratory muscles to the ventilatory response during the test. Whether IMT may alter the respiratory

muscle contribution to ventilation during heavy constant load exercise warrants further investigation.

CHAPTER 7 – GENERAL DISCUSSION

In younger adults the research aims were to evaluate whether:

(I) The respiratory muscles contribute to exercise-induced increases in systemic cytokines and oxidative stress.

(II) An increase in systemic cytokines and oxidative stress was related to diaphragmatic fatigue.

(III) IMT attenuates systemic cytokines and/or oxidative stress during whole-body exercise or a volitional mimic at rest of steady-state (HYPEX) and heavy exercise (VH) hyperpnoea.

(IV) IMT changes an estimation of the maximum lactate steady-state and respiratory muscle recruitment patterns during the lactate minimum test.

In older adults the research aims were to evaluate whether:

(V) IMT attenuates systemic cytokines and/or oxidative stress at rest.

The primary findings in younger adults were:

(I) Plasma IL-6 concentrations increased during EX, HYPEX and VH and plasma IL-1β increased during VH. Plasma interleukin-1 receptor antagonist concentration and oxidative DNA damage to PBMC remained unchanged during VH.

(II) The increase in IL-6 and IL-1β during VH was not related to the induction of diaphragmatic fatigue.

(III) Following IMT, plasma IL-6 concentrations were reduced by 33% during EX, 24% during VH, but were unchanged during HYPEX.

(IV) Following IMT, an estimation of the maximum lactate steady-state and respiratory muscle recruitment patterns remained unchanged during the lactate minimum test.

The primary findings in younger adults were:

(V) Following IMT, nine plasma cytokines and DNA damage to PBMC remained unchanged.

7.0 CONTRIBUTION OF THE RESPIRATORY MUSCLES TO EXERCISE-INDUCED SYSTEMIC CYTOKINES

Volitional hyperpnoea at rest was utilised to address the question of whether respiratory muscles release cytokines during exercise. In this model, validity is improved by mimicking the breathing and respiratory muscle recruitment patterns experienced during whole-body exercise (Babcock et al. 1995; Klas and Dempsey 1989).

Using this model, this thesis presents novel evidence that the respiratory muscles contribute to exercise-induced increases in plasma cytokines. There was an increase in plasma IL-6 concentration following a volitional mimic at rest of the breathing and respiratory muscle recruitment patterns experienced during steady-state (Chapter 3) and heavy (Chapter 4) whole-body exercise. In Chapter 3 pre-intervention peak plasma IL-6 concentration was 2−3 $pg \cdot mL^{-1}$ at 1 to 2 h after volitional hyperpnoea at rest that was commensurate with steady-state exercise. This change represented a 300 to 400% increase above resting values. The magnitude of this increase in plasma IL-6 concentration is comparable with Vassilakopoulos et al. (2002) who reported peak plasma concentrations of ~1.6 $pg \cdot mL^{-1}$ 30 to 120 min after IRL at ~75% MIP until task failure. In contrast, in Chapter 4 a much larger increase

in plasma IL-6 concentration was observed 2 h after volitional hyperpnoea. Pre-intervention peak plasma IL-6 concentration was 4−5 $pg \cdot mL^{-1}$, representing a 600 to 700% increase above resting values. This finding suggests that IL-6 production in the respiratory muscles is dependent upon the intensity of respiratory muscle work. In Chapter 3 mean pre-intervention PTP_{di} (diaphragmatic force output) during volitional hyperpnoea was 700−800 $cmH_2O \cdot s \cdot min^{-1}$ and mean PTP_e (total inspiratory muscle force output) was 400−500 $cmH_2O \cdot s \cdot min^{-1}$. Contrastingly, in Chapter 4, PTP_{di} and PTP_e were increased to 800−900 and 600−700 $cmH_2O \cdot s \cdot min^{-1}$, respectively. Figure 7.1 shows a moderate to strong positive correlation for individual plasma IL-6 increase during the volitional mimic of steady-state (Chapter 3; Volitional hyperpnoea A) and heavy (Chapter 4; Volitional hyperpnoea B) exercise hyperpnoea against respiratory muscle force output and ventilation.

Since the duration of volitional hyperpnoea was the same in both chapters (1 h), it demonstrates that with a greater amount of respiratory muscle work, there are larger increases in plasma IL-6 concentration. This has been previously shown with whole-body exercise. Scott et al. (2011) reported that plasma IL-6 concentration immediately after 1 h of treadmill running was higher at an intensity of 75% $\dot{V}O_{2max}$ compared to intensities of 65 or 55% $\dot{V}O_{2max}$. Furthermore, although not statistically significant the plasma IL-6 concentration was higher at 65% compared to 55% $\dot{V}O_{2max}$.

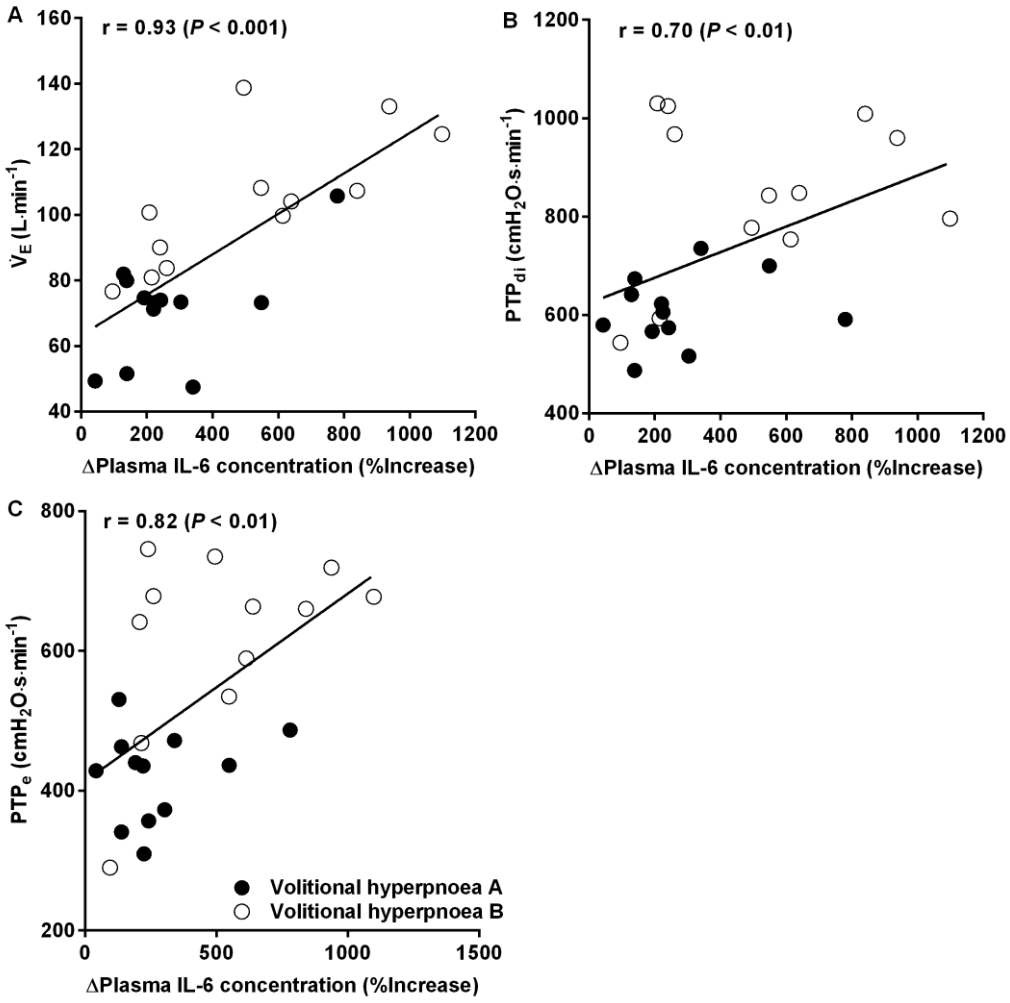


Figure 7.1 Multiple regression analyses for repeated observations within participants (Bland and Altman 1995) where plasma interleukin-6 (IL-6) concentration (peak percentage increase from resting values) was treated as the dependent variable, minute ventilation ($\dot{V}_E$; A); diaphragmatic force output (PTP_{di}; B); and total inspiratory muscle force output (PTP_e; C) during a volitional mimic of steady-state exercise hyperpnoea (Chapter 3; Volitional hyperpnoea A) and heavy exercise hyperpnoea (Chapter 4; Volitional hyperpnoea B) as the independent variables, and participant as the categorical variable. The regression lines are for group mean data.

Alternatively, it could be speculated that the greater increase in plasma IL-6 concentration observed in Chapter 4 was due to a greater contribution of IL-6 from the less efficient accessory inspiratory muscles. Using the a–fv difference technique, Helge et al. (2011) observed that during 90 min of combined arm and leg exercise (60% of $\dot{V}O_{2max}$) the arms produce greater amounts of IL-6 than the legs, even when

relative to the size of the muscle group used. No correlations were present between IL-6 release and exogenous substrate uptake suggesting that differences in muscle morphology may be the cause of the greater IL-6 release in the arms. In healthy males, there are a greater proportion of type II fibres in the vastus lateralis than the biceps brachii (Miller et al. 1993). The same can be observed in the diaphragm compared to the internal intercostals (Figure 1.6; Mizuno and Secher 1989).

Plasma IL-1β and IL-1ra concentrations were only measured in Chapter 4. The decision to only measure these cytokines after this breathing challenge was based on the observation that only very prolonged exercise with primarily an eccentric component results in a small increase in plasma IL-1β concentration (Ostrowski et al. 1999). Therefore, very high levels of respiratory muscle work would be required to elicit a significant response. In Chapter 4, although peak plasma IL-1β concentration was relatively low, it significantly increased from 0.1−0.13 $pg \cdot mL^{-1}$ at rest to 0.27−0.3 $pg \cdot mL^{-1}$ at 1 h in recovery from volitional hyperpnoea. This increase represented a 120 to 130% increase above resting values which is comparable with Vassilakopoulos et al. (2002) who also observed an increase in the plasma IL-1β concentration from ~0.3 $pg \cdot mL^{-1}$ at rest to ~0.8 $pg \cdot mL^{-1}$ 120 min in recovery from IRL at ~75% MIP until task failure (Figure 1.26).

An increase in plasma IL-1ra concentration was not observed after volitional hyperpnoea in Chapter 4. The only known biological role of IL-1ra is to inhibit signalling transduction of IL-1 through the IL-1 receptor complex (Dinarello 2000). It has been reported that IL-1ra is also upregulated by IL-6 and that acute exercise induces an anti-inflammatory environment in the post-exercise period (Steensberg et al. 2003). Therefore, theoretically, the increases in plasma IL-6 and IL-1β concentration should lead to an increase in plasma IL-1ra concentration. The likely

explanation for this finding is the time course of plasma IL-6 and IL-1β concentration in Chapter 4. Following prolonged and strenuous whole-body exercise, others have reported an increase in plasma IL-1ra concentration similar to the magnitude of the plasma IL-6 response, but with peak concentrations recorded between 2–4 h post-exercise (Ostrowski et al. 1999; Scott et al. 2011; Yfanti et al. 2012). The peak concentrations of plasma IL-6 and IL-1β in Chapter 4 after the end of volitional hyperpnoea were observed at +2 and +1 h, respectively. This is consistent with IRL (Vassilakopoulos et al. 2002), but in contrast to whole-body exercise where the plasma concentrations of these cytokines peak immediately post-exercise (Ostrowski et al. 1998; Ostrowski et al. 1999). It is probable that this delay, and the relatively low concentrations of plasma IL-6 and IL-1β (compared to prolonged and strenuous exercise), resulted in plasma IL-1ra not being observed in the systemic circulation until ~2 h following the peak concentration; possibly at ~3–4 h post volitional hyperpnoea, and at a time point was not measured.

7.1 THE ROLE OF BREATHING-INDUCED CYTOKINES AND/OR OXIDATIVE STRESS IN DIAPHRAGM FATIGUE

This thesis presents novel information regarding the role of breathing-induced cytokines and/or oxidative stress in the aetiology of diaphragm fatigue. The role of diaphragm fatigue was assessed because it was suggested that the increase in plasma cytokines may be due to inspiratory / diaphragmatic muscle fibre injury / fatigue since task failure also occurred following IRL (Vassilakopoulos et al. 1999; Vassilakopoulos et al. 2002). Furthermore, the stimuli for breathing-induced cytokines have been reported to be oxidative stress which can also contribute to diaphragmatic fatigue. Diaphragm fatigue was assessed using a non-volitional nerve stimulation technique in Chapters 3 and 4. Diaphragm fatigue was defined as a >15%

reduction in the P_{ditw} response to BAMPS compared to the baseline value (Kufel et al. 2002). Diaphragm fatigue was evident following volitional hyperpnoea in Chapter 4, but not in Chapter 3. In Chapter 4 it was shown that the increase in plasma IL-β concentration occurred with, but was not specifically related to the induction of diaphragmatic fatigue. This finding may indicate that inspiratory / diaphragmatic muscle fibre injury may have occurred after the high level of respiratory muscle work. The biological role of IL-β is poorly understood, but within the respiratory muscles IL-1β may reduce muscle contractility and mediate muscle injury. In both Chapters 3 and 4 there was an increase in plasma IL-6 concentration. IL-6 may be produced and released from the contracting muscles, signalling to the liver to increase its glucose output, and maintain substrate homeostasis. This should prevent an extreme fall in the blood glucose concentration. It could be speculated that the large increase in plasma IL-6 concentration observed in Chapter 4 suggests that there was a reduction in respiratory muscle glycogen content and this also contributed to diaphragmatic fatigue.

Reports have demonstrated that respiratory muscle contractile properties are altered by oxidative stress. A decrease in muscle contractility has been observed *in vitro* by incubating unfatigued isolated muscle fibres with ROS (Lawler et al. 1997) and NO (Lawler and Hu 2000). Conversely, contractile performance is improved by administrating antioxidants during fatiguing contractions *in vitro* (Diaz et al. 1994; Khawli and Reid 1994; Shindoh et al. 1992) and *in vivo* (Kelly et al. 2009b; Supinski et al. 1995; Supinski et al. 1997; Travaline et al. 1997b). In Chapter 4 it was observed that the high level of respiratory muscle work did not alter a marker of systemic oxidative stress. The comet assay was used to determine levels of systemic oxidative stress to DNA damage in PBMC. The modified comet assay can measure

oxidative damage with the use of bacterial repair endonucleases with appropriate specificities that can readily be incorporated into the modified comet assay protocol. This finding suggests that in humans undertaking a high level of respiratory muscle work, systemic oxidative stress is not evident with diaphragm fatigue using this marker. Oxidative DNA damage to PBMC will occur if there is an excessive production of free radicals, inefficient antioxidant defences and/or DNA repair mechanisms. However, whether there was a local increase in diaphragmatic oxidative stress cannot be excluded. Alternatively, the finding in Chapter 4 might suggest that this local production does not spill out to cause sufficient systemic DNA damage in PBMC.

7.2 THE EFFECT OF IMT ON PLASMA IL-6 DURING INCREASED RESPIRATORY MUSCLE WORK

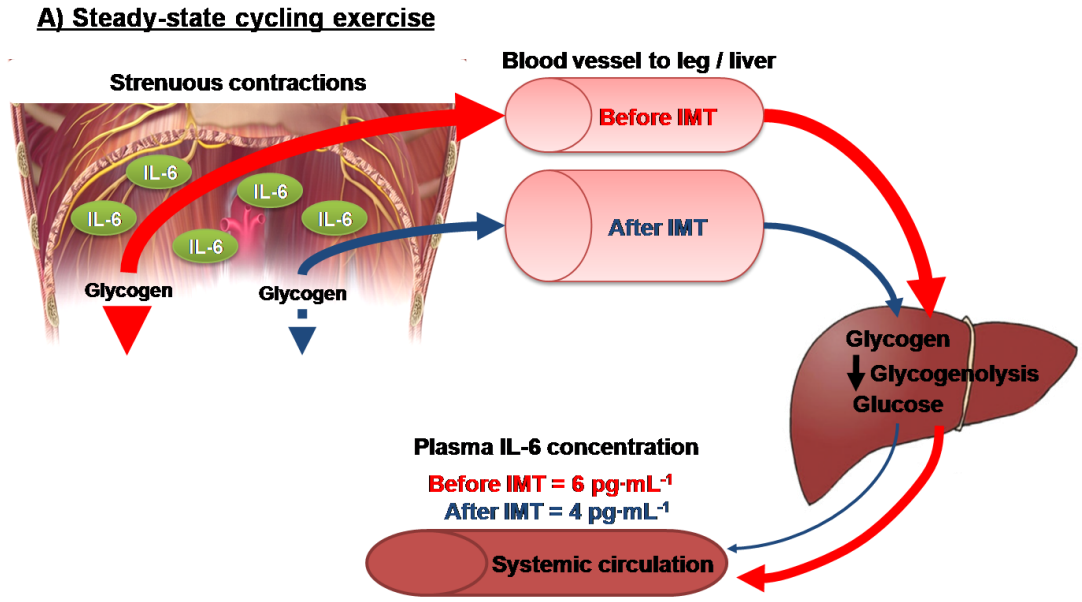
This thesis presents novel information that IMT attenuates plasma IL-6 concentration during exercise (Chapter 3) and following a volitional hyperpnoea mimic at rest of the breathing and respiratory muscle recruitment patterns experienced during heavy (Chapter 4), but not steady-state (Chapter 3) whole-body exercise. Three questions are raised from this finding. Firstly, why is plasma IL-6 concentration attenuated? Changes in inspiratory muscle morphology may explain, in part, the attenuated increase in plasma IL-6 concentration following IMT. There may be post-IMT increases in basal levels of IL-6rα (Akerstrom et al. 2009; Keller et al. 2005), or an increase in antioxidant enzymes (Yfanti et al. 2012). More likely, IMT results in an increase in glycogen synthase within the respiratory muscles and the trained muscles will consequently store more muscle glycogen. It has been previously reported that endurance training in rats increases diaphragmatic muscle glycogen content (Green et al. 1988). Furthermore, muscle glycogen content is

associated with lower IL-6 mRNA expression during acute lower-body exercise (Fischer et al. 2004b). During high levels of respiratory muscle work the untrained muscle may be highly dependent on glycogen as a substrate whereas training leads to increased β-oxidating enzymes and an enhanced capability to oxidise fat and hence to use fat as a substrate during respiratory muscle work. The trained respiratory muscle therefore uses less glycogen during muscular contractions. The activation of muscle IL-6 is glycogen dependent. During prolonged and strenuous respiratory muscle work there is a decrease in respiratory muscle glycogen content, and IL-6 is upregulated at a faster rate. More IL-6 is produced at the same relative work compared with conditions with high muscle glycogen. During respiratory muscle work this results in a lower plasma IL-6 concentration for trained compared to untrained muscles.

The second question regards why plasma IL-6 concentration is attenuated following IMT during exercise, but not a volitional mimic of the exercise hyperpnoea? This finding could be explained by the following hypothesis shown in Figure 7.2. During cycling exercise at an estimated MLSS (Chapter 3) the rate at which skeletal muscle (leg and respiratory) IL-6 is produced is low because skeletal muscle glycogen content is only slightly decreased. There is competition between the leg, respiratory muscles and the liver for blood flow. After IMT there is only a small decrease in the rate of IL-6 production within the respiratory muscles because respiratory muscle glycogen content is only slightly decreased. IMT possibly increases blood flow to the leg muscles (Bailey et al. 2010; Chiappa et al. 2008; McConnell and Lomax 2006; Witt et al. 2007) and/or potentially the liver. Additional blood flow to the legs would increase O_2 (Bailey et al. 2010) and/or glucose delivery (Ebeling et al. 1993) potentially reducing muscle glycogen

utilisation, and blood flow to the liver would increase hepatosplanchnic uptake of IL-6 (Febbraio et al. 2003). Therefore, plasma IL-6 concentration is decreased (Figure 7.2A).

During a volitional mimic at rest of the steady-state exercise hyperpnoea (Chapter 3) the rate at which respiratory muscle IL-6 is produced is low because skeletal muscle glycogen content is only slightly decreased. There is no competition between the leg, respiratory muscles and the liver for blood flow, and the rate of hepatosplanchnic uptake of IL-6 is maintained. After IMT there is only a small decrease in the rate of IL-6 production within the respiratory muscles because respiratory muscle glycogen content is only slightly reduced. Therefore, plasma IL-6 concentration is unchanged (Figure 7.2B).



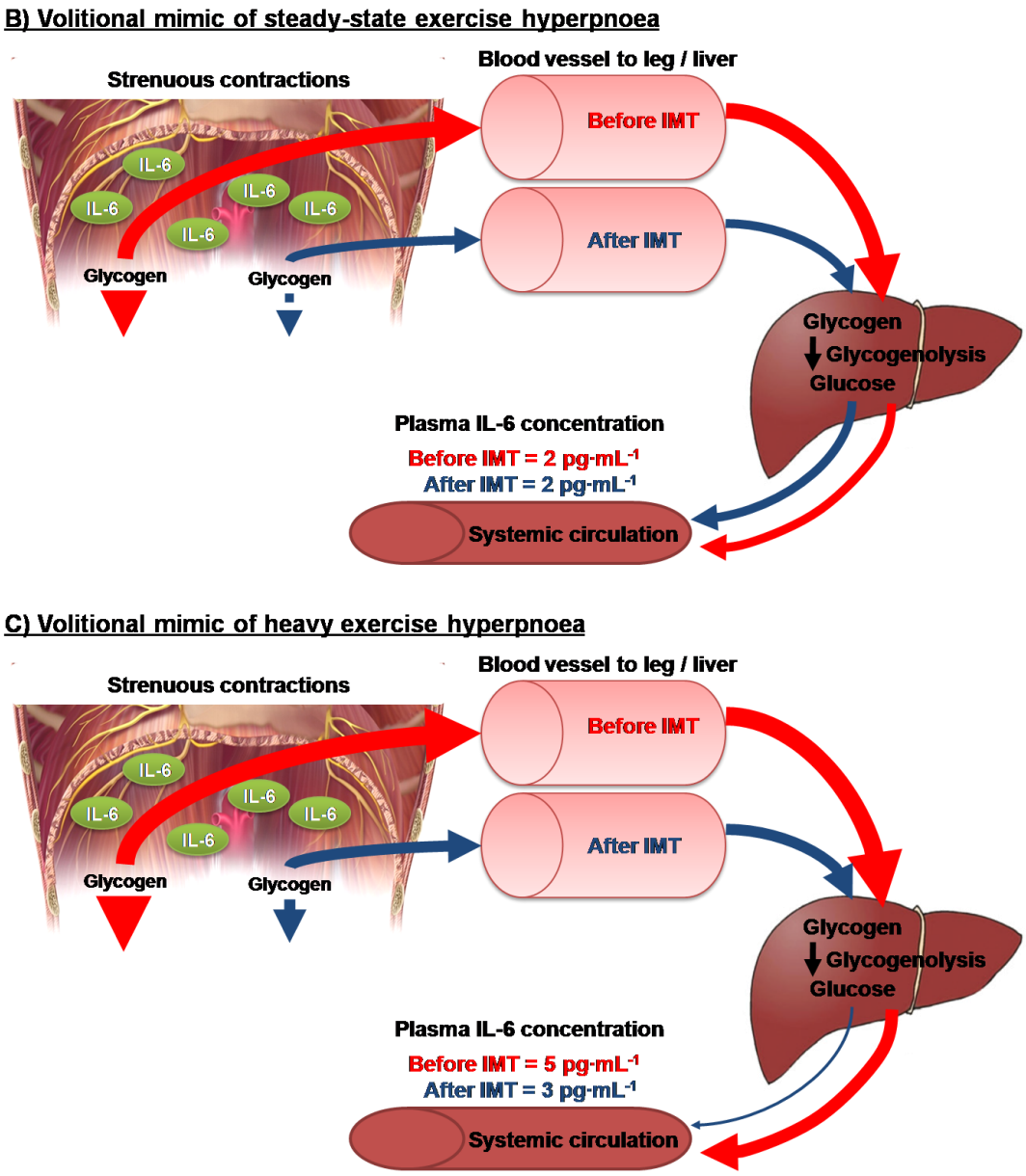


Figure 7.2 Interleukin-6 (IL-6) production and release during steady-state cycling exercise (A), a volitional mimic of steady-state exercise hyperpnoea (B) and a volitional mimic of heavy exercise hyperpnoea (C) before and after inspiratory muscle training (IMT). The activation of skeletal muscle IL-6 is glycogen dependent and during prolonged and strenuous respiratory muscle work there is a decrease in respiratory muscle glycogen content and IL-6 is upregulated. IL-6 is released in an endocrine like manner into the systemic circulation and nearly half is cleared by the liver where glucose output is stimulated. Plasma IL-6 concentration is measured in the systemic circulation. After IMT there is an increase in β-oxidating enzymes within the respiratory muscles, an enhanced capability to oxidise fat, and to use fat as a substrate during respiratory muscle work. The trained respiratory muscles, therefore, uses less glycogen during muscular contractions.

The third question leads on from this point; why is a training effect revealed with a higher level of respiratory muscle work as plasma IL-6 concentration is attenuated during a volitional mimic at rest of the breathing and respiratory muscle recruitment patterns experienced during heavy whole-body exercise in Chapter 4? This suggests that there may be a threshold above which an effect of IMT is observed. During a volitional mimic of heavy exercise hyperpnoea (Chapter 4) the rate at which respiratory muscle IL-6 is produced is high because there is a significant reduction in respiratory muscle glycogen content. There is no competition between the leg, respiratory muscles and the liver for blood flow. However, at this point the capacity for hepatosplanchnic IL-6 uptake cannot match the rate of respiratory muscle IL-6 release (Febbraio et al. 2003). After IMT, there is large decrease in the rate of IL-6 production within the respiratory muscles because respiratory muscle glycogen content is only slightly reduced. Therefore, plasma IL-6 concentration is decreased (Figure 7.2C).

7.3 WIDER SIGNIFICANCE OF FINDINGS AND FUTURE PERSPECTIVES

These findings demonstrate that the respiratory muscles contribute to exercise-induced increases in plasma cytokines. The first requirement for future work exploring this area is to determine whether decreased glycogen content is the stimulus for IL-6 production from respiratory muscles. This could be achieved by depleting respiratory muscle glycogen content using IRL and/or volitional hyperpnoea the day before replicating the experimental trials described in the experiment chapters of this thesis.

The response of plasma IL-6 and TNF-α concentration from COPD patients during moderate intensity exercise is greater than the response observed in healthy

controls (Rabinovich et al. 2003). The inspiratory muscles of COPD patients experience increased workloads, due to airflow limitation, hyperinflation, and a reduction in inspiratory muscle contractile properties. Therefore, it could be speculated that the dysregulated cytokine response to exercise is due to a local production of respiratory muscle cytokines that then spill out into the systemic circulation. Thus, future perspective work would examine whether the respiratory muscles of COPD patients contribute to the dysregulated cytokine response to exercise that is observed.

COPD patients also experience systemic inflammation which can account for a large part of the morbidity and mortality observed (Sin et al. 2006). There are two opposing views regarding the source of the systemic inflammation in COPD patients. Some suggest that inflammatory markers spill out from the lungs of patients into the systemic circulation whereas others have suggested that that multiple organs contribute (Barnes and Celli 2009). It is still unknown whether the respiratory muscles contribute to this systemic inflammation. However, Casadevall et al. (2007) reported that the external intercostals of COPD patients had higher mRNA and protein expression of TNF-α, IL-1β and IL-6 (although IL-6 mRNA was not significant) compared to aged-matched healthy controls. Thus, it could be speculated that they do. Future work would also examine whether IMT can attenuate local cytokine production within the inspiratory muscles of COPD patients.

Lastly, the relationship between oxidative stress and cytokines on the aetiology of diaphragm fatigue are still poorly understood. The findings observed in Chapters 3 and 4 demonstrated that increases in plasma IL-6 and IL-1β concentrations can occur with, but are not related to, the induction of diaphragm fatigue. Furthermore, there were no effects of high levels of respiratory muscle work

on DNA damage in PBMC. Contractile performance is improved by administrating antioxidants before fatiguing contractions *in vivo* (Kelly et al. 2009b; Supinski et al. 1995; Supinski et al. 1997; Travaline et al. 1997b). One study has utilised objective nerve stimulation techniques, but IRL was used as a model for a breathing challenge to induce diaphragm fatigue (Figure 30; Travaline et al. 1997a). Alternatively, Kelly et al. (2009b) used cycling exercise at 85% $\dot{V}O_{2peak}$ until volitional tolerance a more ecologically valid model, but diaphragm fatigue was assessed using a volitional measure of MIP. Thus, a future study would examine the effects of antioxidant administration on exercise tolerance and objectively measure diaphragm fatigue with bilateral phrenic nerve stimulation.

7.4 THE EFFECT OF IMT ON SYSTEMIC PLASMA CYTOKINES AND/OR OXIDATIVE STRESS OBSERVED IN AGEING

Chapter 5 provided novel evidence in older adults using a rigorous, placebo controlled design with an adequate sample size that IMT can increase diaphragm thickness and dynamic inspiratory muscle function, but does not change resting markers of systemic inflammation (plasma cytokines) and oxidative stress (DNA damage in PBMC). Whilst IMT did not change resting markers of systemic inflammation and oxidative stress, it cannot be excluded whether IMT attenuated local diaphragmatic cytokines and/or oxidative stress. A diaphragmatic or intercostal biopsy would allow this area to be explored. However, this is a very invasive measurement that requires open surgery or thoracscopy under general anaesthesia and for these healthy individuals this would not be ethical. It could be that the improvement in inspiratory muscle function and structure did not reach a threshold necessary to achieve a potential reduction in systemic cytokines and/or oxidative stress. Previous studies have shown that whole-body resistance or endurance training

can decrease resting systemic cytokine concentrations (Kohut et al. 2006; Nicklas et al. 2008; Phillips et al. 2010; Prestes et al. 2009) and markers of oxidative stress (Fatouros et al. 2004; Parise et al. 2005; Takahashi et al. 2012). However, the respiratory muscles only weigh ~960 g (Freedman et al. 1983) and represent ~3% of total body mass (Robertson et al. 1977). Thus, IMT only targets a small muscle group and although they do contribute to systemic cytokines concentrations during increased respiratory muscle work, this does not change under resting conditions.

In contrast, the effects of IMT on the cytokine response to intense exercise and/or volitional hyperpnoea in older adults would be ethical. Older participants were unable to undertake intense exercise and/or volitional hyperpnoea in the present thesis as the study being the first of its type at the University. In future, this type of study may be available based on the outcomes of Chapter 5.

7.5 THE EFFECT OF IMT ON THE LACTATE MINIMUM TEST AND ON THE CONTRIBUTION OF THE RESPIRATORY MUSCLES TO THE VENTILATORY RESPONSE

Chapter 6 provided novel evidence that IMT does not change the estimation of MLSS using the lactate minimum test and it does not change the contribution of the respiratory muscles to the ventilatory response during the test. This type of test may not be suitable to demonstrate IMT-mediated adaptations in the contribution of the respiratory muscles to the ventilatory response. The lactate minimum test involves an incremental test performed until exhaustion, a short active recovery phase where the ventilatory response does not decrease to resting levels, and a sub-maximal incremental phase where ventilation progressively increases, but only reaches near-maximal levels in the final stage. During constant load (Babcock et al. 1995; Babcock et al. 1995; Babcock et al. 1996; Babcock et al. 1998; Babcock et al.

2002; Johnson et al. 1993; Mador et al. 1993) or maximal incremental (Romer et al. 2007) exercise until the limit of tolerance there is a progressive increase in the contribution from the less efficient accessory inspiratory (and expiratory) muscles, and a decrease in the contribution from the diaphragm to the ventilatory response. When the inspiratory muscles are unloaded with a PAV respiratory muscle recruitment patterns remain at resting values during heavy constant load exercise (Babcock et al. 2002), but remain unchanged during maximal incremental exercise (Romer et al. 2007). These findings suggest that reducing the WOB of the inspiratory muscles during maximal incremental exercise does not alter respiratory muscle recruitment patterns. It may be that during the lactate minimum test there is an insufficient accumulation of metabolic stimuli and/or diaphragmatic fatigue to make any RMT-mediated adaptations evident. Future work would examine whether IMT may alter the respiratory muscle contribution to ventilation during heavy constant load exercise test until the limit of tolerance.

7.6 CONCLUSION

In conclusion, this thesis provides novel evidence in younger adults that the respiratory muscles contribute to exercise-induced increases in plasma IL-6 and IL-1β concentrations and that this increase is not related to diaphragmatic fatigue. IMT attenuates plasma IL-6 concentration during exercise in younger adults, but not in a range of plasma cytokines at rest in older adults. It is attractive to speculate that the respiratory muscles contribute to the systemic inflammation observed in COPD patients and IMT may reduce the dysregulated cytokine response observed during exercise of COPD patients.

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APPENDIX 1 – YOUNGER PARTICIPANT SELF-REPORTING MEDICAL QUESTIONNAIRE

Health screen

NOTTINGHAM TRENT UNIVERSITY

HEALTH SCREEN

Name or Number .....

Please complete this brief questionnaire to confirm fitness to participate:

1.	**At present,** are you:		
(a)	on medication, prescribed or otherwise (including supplements or antioxidants)	Yes ☐	No ☐
(b)	attending your general practitioner	Yes ☐	No ☐
(c)	on a hospital waiting list	Yes ☐	No ☐
2.	**In the past two years,** have you had any illness which require you to:		
(a)	consult your GP	Yes ☐	No ☐
(b)	attend a hospital outpatient department	Yes ☐	No ☐
(c)	be admitted to hospital	Yes ☐	No ☐
3.	**Have you ever** had any of the following?		
(a)	Convulsions/epilepsy	Yes ☐	No ☐
(b)	Asthma	Yes ☐	No ☐
(c)	Eczema	Yes ☐	No ☐
(d)	Diabetes	Yes ☐	No ☐
(e)	A blood disorder	Yes ☐	No ☐
(f)	Head injury	Yes ☐	No ☐
(g)	Digestive problems	Yes ☐	No ☐
(h)	Heart problems	Yes ☐	No ☐
(i)	Problems with bones or joints	Yes ☐	No ☐
(j)	Disturbance of balance / coordination	Yes ☐	No ☐
(k)	Numbness in hands or feet	Yes ☐	No ☐
(l)	Disturbance of vision	Yes ☐	No ☐
(m)	Ear / hearing problems	Yes ☐	No ☐
(n)	Thyroid problems	Yes ☐	No ☐
(o)	Kidney or liver problems	Yes ☐	No ☐
(p)	Allergy to nuts, alcohol etc	Yes ☐	No ☐
(q)	Susceptibility to nosebleeds	Yes ☐	No ☐
4.	**Has any,** otherwise healthy, member of your family under the age of 35 died suddenly during or soon after exercise?	Yes ☐	No ☐
5.	Are there any reasons why blood sampling may be difficult?	Yes ☐	No ☐
6.	Have you had a blood sample taken previously?	Yes ☐	No ☐
7.	Have you had a cold or flu or any flu like symptoms in the last month?	Yes ☐	No ☐
8.	Do you have a cardiac pacemaker fitted or other im-planted electrical devices or metal parts in your body?	Yes ☐	No ☐
9.	Are you currently or previously a smoker?	Yes ☐	No ☐

APPENDIX 2 – ETHICAL APPROVAL FOR EXPERIMENTAL CHAPTERS 3, 4 AND 6

NOTTINGHAM TRENT UNIVERSITY

COLLEGE OF SCIENCE AND TECHNOLOGY

ETHICAL COMMITTEE (HUMANS)

Application No:	(113) Amendment
Title:	The Effects of the Work of Breathing on Blood Borne Inflammatory Markers
Applicants:	Dr Michael Johnson
Date of Meeting:	25 May 2010

With regard to your application to the recent meeting of the Ethical Committee (Humans), the Committee have reviewed this and the following action has been decided:

☑ **Approved by Chair's Action**

☐ Approved, subject to the following information being provided/clarified:

☐ Decision deferred, pending receipt of further information:

☐ Not Approved

Professor Bob Rees
Chair
Ethical Review Committee (Humans)

1/06/10
Date

NOTTINGHAM TRENT UNIVERSITY

APPENDIX 3 – AMENDMENT FOR ETHICAL APPROVAL FOR EXPERIMENTAL CHAPTERS 3, 4 AND 6

NOTTINGHAM TRENT UNIVERSITY

COLLEGE OF SCIENCE AND TECHNOLOGY

ETHICAL COMMITTEE (HUMANS)

Application No:	(113)
Title:	The Effects of Breathing on Blood Borne Inflammatory Markers
Applicants:	Dr Mike Johnson
Date of Meeting:	24 February 2010 (Chair's Action)

With regard to your application to the Ethical Committee (Humans), the Committee have reviewed this and the following action has been decided:

☑ **Approved (by Chair's Action)**

☐ Approved, subject to the following:

☐ Not Approved

.....

Professor Bob Rees
Chair
Ethical Review Committee (Humans)

26/02/10
.....
Date

APPENDIX 4 – EXCLUSION CRITERIA USED TO DEFINE "HEALTHY" OLDER PARTICIPANTS FOR EXERCISE STUDIES (GREIG ET AL. 1994)

- History of myocardial infarction within the previous 10 years.
- Cardiac illness: e.g. symptoms of aortic stenosis, acute pericarditis, acute myocarditis, aneurysm, severe angina, clinically significant valvular disease, uncontrolled dysrrhythmia, claudication, within the previous 10 years
- Thrombophlebitis or pulmonary embolus within the previous 10 years
- History of cerebrovascular disease
- Acute febrile illness within the previous 6 months
- Moderate or severe airflow obstruction
- Metabolic disease (e.g. diabetes, thyroid disease) whether controlled or uncontrolled
- Major systemic disease diagnosed or active within the previous 20 years (e.g. cancer, rheumatoid arthritis)
- Significant emotional distress, psychotic illness or anything worse than mild anxiety or depression, within the previous 10 years
- Osteoarthritis, classified by inability to perform maximal contractions of upper and lower limbs without pain.
- Bone fracture sustained within previous 2 years.
- "Old person's fracture" after 40 years of age (wrist, hip, vertebral)
- Non-arthroscopic joint surgery, ever, in the relevant limb part.
- Any reason for loss of mobility for greater than 1 week in the previous 6 months or greater than 2 weeks in the previous year
- On daily medication (including daily aspirin or any medication with similar effects); on a oestrogen replacement therapy; antioxidants or supplements; on medication for hypertension, or with a diuretic for any other reason, even if not daily
- Obese, i.e. a Quetelet index (wt.ht-2) greater than 29.9
- Resting systolic blood pressure > 200mmHg, or resting diastolic blood pressure > 100mmHg
- Unable to walk unsupported

APPENDIX 5 – ETHICAL APPROVAL FOR EXPERIMENTAL CHAPTER 5

NOTTINGHAM TRENT UNIVERSITY

COLLEGE OF SCIENCE AND TECHNOLOGY

ETHICAL COMMITTEE (HUMANS)

Application No:	**166**
Title:	The effects of inspiratory muscle training on blood-borne inflammatory markers in the elderly.
Applicants:	Dr Michael Johnson, Dr Graham Sharpe and Mr Dean Mills
Date of Meeting:	8 June 2011

With regard to your application to the recent meeting of the Ethical Committee (Humans), the Committee have reviewed this and the following action has been decided:

- [x] **Approved**
- [ ] Approved, subject to the following information being provided/clarified:
- [ ] Decision deferred, pending receipt of further information:
- [ ] Not Approved

Professor Bob Rees
Chair
Ethical Review Committee (Humans)

Date: 9/12/11

APPENDIX 6 – COMMITMENT LETTER FROM TRAINED CARDIOLOGIST TO INTERPRET ELECTROCARDIOGRAMS

Nottingham University Hospitals NHS
NHS Trust

Please ask for: Dr Will's Smith Secretary

CITY HOSPITAL CAMPUS
Trent Cardiac Centre
Hucknall Road
Nottingham
NG5 1PB

Ref: WHS/GS

Tel: 0115 969 1169 ext 56294
Fax: 0115 9627691

03 August 2011

Mr Dean Mills
School of Science and Technology
Nottingham Trent University
Burton Street
Nottingham
NG1 4BU

Dear Mr Mills

Re: The effects of inspiratory muscle training on bloodborne inflammatory markers in the elderly

I would be happy to interpret screening electrocardiograms on patients enrolled in this study. As we discussed it would be helpful to see their medical questionnaires alongside the ECG's so that if there are abnormalities I can begin to put this into some context. I would also need the patient's address so that I could communicate directly with the patient to recommend a visit to their GP and possible onward referral if more concerning abnormalities are identified.

Kind regards

Yours sincerely,

Dr Will Smith
Consultant Cardiologist

Chairman: Dr Peter Barrett Chief Executive: Dr Peter Homa

Trust HQ: North Road, City Hospital campus, Hucknall Road, Nottingham, NG5 1PB

NUH010095