THE ROLE OF THE L-ARGININE/NITRIC OXIDE PATHWAY IN THE ARTERIAL ADAPTATION TO SIMULATED MICROGRAVITY

by

SIMON RODERICK HUTCHINGS

M.Pharmacol., The University of Bath, 2003

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

in

THE FACULTY OF GRADUATE STUDIES

(Pharmacology and Therapeutics)

THE UNIVERSITY OF BRITISH COLUMBIA

Vancouver

September 2008

ABSTRACT

Orthostatic intolerance following exposure to simulated or actual microgravity is observed following spaceflight and extended periods of bed rest, and is not always associated with simultaneous hypotension. Differential adaptation of cephalic and caudal arterial vasculatures (as a result of removal of the normal hydrostatic gradient) is proposed as a potential mechanism underlying this phenomenon. A potential role for changes to the L-arginine/nitric oxide pathway in such adaptations has been suggested, predominantly from previous *in vitro* studies; using an established model of simulated microgravity (head-down tilt; HDT). This thesis investigates whether findings in isolated vessels are reflected by *in vivo* measurements of cephalic and caudal vascular function.

Using carotid or iliac artery flow normalized to mean arterial pressure as an index of cerebral or hind limb vascular conductance, autoregulatory cerebral vasodilatation in response to lower body negative pressure was found to be impaired following HDT. In addition, α_1-adrenoceptor agonist-mediated vasoconstriction was decreased in the cerebral vasculature and increased in the peripheral and hind limb vasculature. Administration of acetylcholine or the non-selective nitric oxide synthase (NOS) inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME) demonstrated a decreased contribution of NOS to cerebrovascular tone, but an increased contribution of NOS to peripheral vascular resistance and tone of the hind limb vasculature. Together with a lack of difference in the response to the selective inducible NOS (iNOS) inhibitor 1400W, these results suggest that differential adaptation of eNOS may account for the observed differences between control and HDT animals.

Further investigation of the changes to the L-arginine/nitric oxide pathway suggest that these changes are not associated with changes in eNOS expression, but may be related to altered

activity of eNOS. Furthermore, the bioavailability (as measured by pharmacokinetic half life) or the vascular effector mechanisms (as measured by the haemodynamic response to exogenously administered nitric oxide) responsible for the effects of nitric oxide were also shown to be unaffected by HDT.

These findings suggest that differential adaptation of the L-arginine/nitric oxide pathway may contribute to the inability to raise total peripheral resistance and impaired cerebral autoregulation following HDT, thereby representing a mechanism of orthostatic intolerance following exposure to microgravity.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ADH	antidiuretic hormone	iNOS	inducible nitric oxide synthase
ADMA	asymmetrical dimethylarginine	IP_3	inositol 1,4,5-trisphosphate
AMT	2-amino-dihydro-6-methyl-4H-1,3-thiazine	K_A	functional dissociation constant
ANOVA	analysis of variance	LBNP	lower body negative pressure
BNP	brain natriuretic peptide	L-NAME	N^{ω}-nitro-L-arginine methyl ester
cAMP	cyclic adenosine monophosphate	MAP	mean arterial pressure
$C_{carotid}$	vascular conductance distal to the common carotid artery	nNOS	neuronal nitric oxide synthase
cGMP	cyclic guanosine monophosphate	NOS	nitric oxide synthase
C_{iliac}	vascular conductance distal to the common iliac artery	O.D.	optical density
cpm	counts per minute	PBS	phosphate-buffered saline
CRC	concentration-response curve	PDE III	phosphodiesterase III
CSA	cross-sectional area	PGI_2	prostaglandin I_2/prostacyclin
ED_{50}	Potency	PKA	protein kinase A
EDHF	endothelium-derived hyperpolarizing factor	PKG	protein kinase G
EDRF	endothelium-derived relaxing factor	q	fraction of receptors remaining functional
E_{max}	Efficacy	$Q_{carotid}$	common carotid artery blood flow
eNOS	endothelial nitric oxide synthase	Q_{iliac}	common iliac artery blood flow
HDBR	head-down bed rest	sGC	soluble guanylate cyclase
HDT	head-down tilt	SNP	sodium nitroprusside
HLU	hind limb unweighting	SR	sarcoplasmic reticulum
HR	heart rate	TPR	total peripheral resistance

ACKNOWLEDGEMENTS

This thesis represents the endeavours of over four years of work that would have been significantly more challenging (and less rewarding) were it not for the invaluable help and support of several individuals. None more so than my supervisor Dr Catherine Pang, who, through providing an ideal balance of scientific guidance and significant academic freedom from the very first days of my post-graduate research, has contributed to my continued growth as an independent integrative pharmacologist. For this I owe an immense debt of gratitude.

Sincere thanks are also extended to the members of my supervisory committee. Dr Kuo-Hsing Kuo for his thoughtful insights into my research from the perspective of an exceptional anatomist, and to Dr John McNeill for continually encouraging me to question the value of any proposed study in order to maximise their contribution to my work. To Dr Michael Walker I owe particular thanks, for both his infectious passion for pharmacology and his relentless quest to instil sound scientific practices into any young pharmacologists he has contact with.

Establishment of the HDT model in our laboratory is predominantly a result of the generous help and guidance from Dr Susan Jacobs-Kaufmann at the University of Alberta. The technical assistance of Su Lin Lim must also be recognised, whose competence and skill in a wide variety of pharmacological techniques, together with her relentless patience, played a huge part in my technical development in the laboratory. Similarly, the contribution of Christian Caritey to the technical aspects of this research and the development of novel, custom-built apparatus deserves special thanks. I have also been fortunate to work with several graduate student colleagues and undergraduate students, namely Dongzhe Song, Evelyn Kwok, Shane Lloyd and Jong Moo Kim, whose enthusiasm and friendship, in addition to their valuable assistance and contributions to the work presented in this thesis, have made my time at UBC an extremely enjoyable one.

Finally, to a loving and supportive family, thank you for understanding when I couldn't be there, and for being there for me when I needed you.

1. INTRODUCTION

1.1 OVERALL PHYSIOLOGICAL ADAPTATION TO MICROGRAVITY

1.1.1 Overview

Thousands of years of evolution under the influence of the earth's gravitation field have resulted in the physiological adaptation by most bipeds to the forces imparted on the body when in an upright position. With respect to the cardiovascular system, such forces result in a hydrostatic pressure gradient along the cephalocaudal axis, from brain to the feet (~70 to ~200 mmHg in humans) (Gauer & Thron, 1965). Upon exposure to extremely low gravitational forces (microgravity) or assumption of a supine position, mean arterial pressure (MAP) measured at the level of the head, heart and feet equilibrates to ~100 mmHg (Charles & Lathers, 1991; Gauer & Thron, 1965). It is important to note that in the context of this area of research, the term microgravity refers to the reduced gravitational force experienced in space compared with that on earth. The terms 'weightlessness' or 'zero-gravity' are misnomers as in space gravity is not absolutely zero. Microgravity should also not be considered to be exactly 10^{-6} of the gravity on earth (1 μG), as measurements of gravitational forces in the International Space Station show an array of multi-directional accelerations of varying magnitude - an average of these measurements gives an approximate gravitational force experienced in space of a few μG (Nelson & Jules, 2004). Until the first manned spaceflight by the Soviet cosmonaut Yuri Gagarin in 1961 (Rauschenbach *et al.*, 1993), exposure to microgravity in humans was limited to brief periods lasting just a few minutes (if not seconds) experienced by freefalling parachutists or by high performance air force or stunt pilots during manoeuvres involving gravitational forces approaching (or sometimes exceeding) 0 g (Haber & Haber, 1950).

Apart from these brief periods experienced by pilots, the most similar environment to that of microgravity (from a physiological perspective) experienced by most humans prior to the advent of spaceflight was during periods of prolonged bed rest (Pavy-Le Traon *et al.*, 2007). As mentioned above, assumption of a supine position removes the downwards forces experienced by the various systems of the body in an upright position, including the cardiovascular, central nervous and musculoskeletal systems (Gauer & Thron, 1965; Nicogossian *et al.*, 1994). One of the earliest formal observations of the deleterious effects of long-term bed rest was that of the reduction in bone mineral density. Contrary to general medical practice at the time (that assumed bed rest to be a universal remedy for a variety of common ailments) an American physician studying bone loss in polio victims during the 1940s speculated that the increased calcium excretion observed in these patients may be independent of the disease itself, and may actually be due to the prolonged period of immobility whilst being treated. In a simple, yet elegant clinical trial, decreased leg bone density (associated with increased excretion of calcium) was observed following 30 days of bed rest in healthy male volunteers (Dietrich *et al.*, 1948).

The similarities between bed rest and exposure to actual microgravity became apparent during the American *Gemini* program in the 1960s, which involved the longest duration of spaceflight at that time of 14 days. Just as in the earlier bed rest study by Dietrich *et al.* (1948), a loss of lower limb bone density associated with loss of bone calcium was observed to varying degrees in all crew members (Calvin & Gazenko, 1975). An additional observation common to nearly all spaceflights part of the Gemini program longer than 24 hrs in duration was that of *orthostatic intolerance* (Buckey *et al.*, 1996; West, 2000). Defined as the inability to maintain cerebral perfusion upon moving from a supine or seated position to standing (leading to loss of consciousness)(Streeten, 1999), orthostatic intolerance has also been observed in countless

studies on the physiological effects of bed rest (Pavy-Le Traon *et al.*, 2007). This confirmed the speculation by medical and scientific communities that removal of the hydrostatic pressure gradient normally exerted on the cardiovascular system by gravity during exposure to microgravity may induce changes in the cardiovascular system that can pose a hindrance to normal function both during spaceflight, and following return to the 1 g environment of earth (Watenpaugh & Hargens, 1996).

The major initial consequences of the loss of pressure gradient are a shift of fluid from the interstitial space of the splanchnic bed and lower extremities to the blood compartment accompanied by a cephalad (headwards) movement of blood to the thoracic vasculature. Relative to normal conditions, cerebral arterial pressure is increased while lower extremity arterial pressure is decreased (Arbeille *et al.*, 1996; Aubert *et al.*, 2005; Watenpaugh & Hargens, 1996). The net effects of these changes on the cardiovascular system during chronic (greater than 24 hr) periods of microgravity exposure are numerous, and include changes in vascular structure, function, and neural/neurohumoral modulation, in addition to altered control of blood volume (Charles *et al.*, 1994; Johnson, 1979; Zhang, 2001). These adaptations presumably occur to match physiological function with the body's new environment (i.e. microgravity), such is the nature of human physiology (and a large part of our success as a species!). It is therefore inaccurate to think of this phenomenon as a disease *per se*, or even a maladaptation. However when the body is once again subjected to orthostatic stress - either following return to earth from space or recovery from an extended period of bed-rest - such adaptation is no longer appropriate and can result in a compromised compensatory reserve to orthostatic challenge, manifested by pre-syncopal or syncopal episodes (Buckey *et al.*, 1996; Charles *et al.*, 1994; Convertino, 2002).

The current body of research investigating the physiological processes involved in cardiovascular adaptation to microgravity reveals a complex aetiology, with all major components of the cardiovascular system being involved to a certain degree (Aubert *et al.*, 2005; Charles *et al.*, 1994; West, 2000). Furthermore, these changes likely do not occur independently of the non-cardiovascular adaptations that are well characterized following microgravity exposure, including skeletal muscle atrophy (Turner, 2000), bone demineralisation (Fitts *et al.*, 2000), and neurological changes (Edgerton & Roy, 2000). Moreover, as shown in Figure 1-1, different physiological systems adapt over different time courses, which may be divided into three main phases (Watenpaugh & Hargens, 1996):

- *immediate response* (seconds to minutes); e.g. cephalic fluid shift and decreases in central venous pressure
- *short term acclimatisation* (hours to days); e.g. neurovestibular disturbances and decreased plasma volume.
- *long term adaptation* (weeks to months); e.g. skeletal muscle atrophy, bone demineralisation and cardiovascular deconditioning.

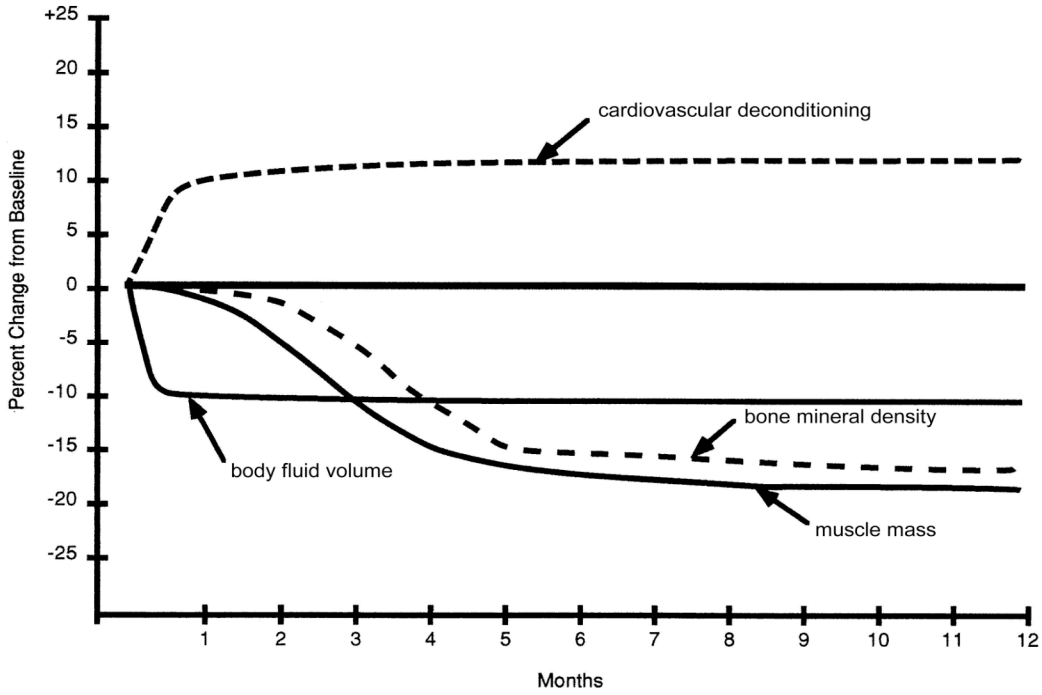

Figure 1-1 - Timeline of changes to major physiological systems in response to microgravity
Approximate percentage change in selected physiological functions in response to exposure to microgravity. Data is compiled from 289 crew members from spaceflights between 1961 and 1996. The duration of spaceflight varies considerably between subjects, with only 46 crew members completing missions longer than 1 month. While extrapolation from this relatively small, statistically biased sample to the general population is problematic, general trends (which are mirrored to a large degree from earth based human and animal simulations) can be observed. For instance, cardiovascular function and body fluid levels begin to change within hours of microgravity exposure, whereas bone demineralization and muscle atrophy do not peak until 4-6 months in space (adapted with kind permission from Nicogossian *et al.*, 1994).

1.1.2 Non-cardiovascular adaptation to microgravity

While the focus of this thesis concerns adaptations specific to the cardiovascular system, it is of importance to briefly consider the nature of the major changes observed in other physiological systems. Contrary to the glamorous impressions portrayed by the media, initial exposure to the microgravity environment of space flight can induce several undesirable symptoms, including facial oedema, back pain, headache, congestion, nausea and vomiting

(Graebe *et al.*, 2004; Williams, 2003). As the duration of spaceflight increases, these acute symptoms largely subside as the body begins to adapt to the new environment. While the initial responses to microgravity may produce some mild undesirable symptoms, the short term acclimatisations and long term adaptations to microgravity pose the greatest risk to the health and normal functioning of crew members during, and particularly following, longer duration spaceflight. In this regard, the physiological systems that are affected by microgravity to the largest extent (exhibiting marked changes in function) are the musculoskeletal, neurovestibular, pulmonary, immune and endocrine systems (Graebe *et al.*, 2004; Nicogossian *et al.*, 1994; West, 2000).

1.1.2.1 Adaptation of the musculoskeletal system

Possibly one of the more pronounced non-cardiovascular adaptations to microgravity occurs in the musculoskeletal system, where the removal of gravitational loading results in both muscle atrophy and a loss of bone mineral density (Turner, 2000). As observed in the aforementioned study of the effects of prolonged bed rest on bone metabolism in healthy individuals in the 1940s (Dietrich *et al.*, 1948), significant bone loss was observed during the 84 day US Skylab mission in 1972. This condition, suggested as being similar in nature to disuse osteoporosis was associated with decreased *os calcis* density, hypercalcaemia, hyperphosphataemia and increased calcium excretion (Johnston & Dietlein, 1977; Schneider *et al.*, 1994). Similar observations have been made in other longer duration missions (Caillot-Augusseau *et al.*, 1998; Grigoriev *et al.*, 1991; Smith *et al.*, 1999), with average bone mineral density losses of 1-2 % per month being reported for load-bearing bones (Bikle *et al.*, 1997; Schneider *et al.*, 1994; Turner, 2000). In addition to an increased risk of fractures from decreased bone strength, the associated hypercalciurea results in a significant risk of renal stone formation (Whitson *et al.*, 2001; Whitson *et al.*, 1999).

Given the effects on load-bearing bones of prolonged exposure to microgravity, it is perhaps reasonable to expect analogous deterioration in skeletal muscle during extended spaceflight - particularly 'anti-gravity' muscle groups used for maintenance of posture that are continually opposing the effects of gravity under normal conditions. Indeed, decreases in muscle mass, force and power have been well documented following longer duration spaceflights (Johnston & Dietlein, 1977; Riley & Ellis, 1983; Rummel *et al.*, 1975). Muscle atrophy and loss in lean body mass, associated with a decline in peak force and power are likely exacerbated by decreases in body fluid volume (Fitts *et al.*, 2000; Leonard *et al.*, 1983). For example, maximal voluntary contractions of the plantar flexor muscle were decreased by 20 – 48 % following a 6 month mission (Fitts *et al.*, 2001). Further to losses in mass and strength, increased muscle fatigue and ataxia are also observed, posing additional impairments on normal function during missions and upon return to earth (Jaweed *et al.*, 1994; Riley, 1999).

It is clear that the effects of prolonged exposure to microgravity on the musculoskeletal system have significant implications for not only normal function during space missions, but to the post-mission recovery process. Compared with the adaptations of other physiological systems to microgravity, the time course of reversal of these adaptations upon return to earth is substantial; the rate of recovery of bone mineral density and muscle mass has been found to be proportional to the duration of microgravity, and progresses more slowly than the original rate of loss (Bikle *et al.*, 1997; Turner, 2000).

1.1.2.2 Adaptation of the neurovestibular system

As with the rest of human physiology, the neurovestibular system has evolved under the stable influence of the earth's gravity. It is therefore not surprising that in a microgravity environment, conflicting afferent information from the eyes, joint and muscle proprioceptors and the vestibular system result in a plethora of clinical features (including disorientation,

dizziness, malaise, nausea and vomiting), collectively know as *space motion sickness* (Reschke *et al.*, 1994). The incidence of this condition ranges from 40 – 70 % of crew members during the first two to three days in space (Davis *et al.*, 1988; Simanonok & Charles, 1994), and despite the symptoms subsiding relatively quickly, the impairment of normal function due to space motion sickness can cause poses a major problem. This is perhaps reflected by the report that drugs to treat the symptoms of space motion sickness constitute ~50% of all medications distributed to crew members (Putcha *et al.*, 1999).

Unlike some of the other physiological adaptations to microgravity, neurovestibular disturbances are not usually observed following prolonged bed rest (Pavy-Le Traon *et al.*, 2007). For this reason, the mechanisms underlying the phenomenon are under-researched relative to adaptations to the musculoskeletal or cardiovascular systems (West, 2000). Based on the similarity of the symptoms exhibited by crew members to space motion sickness with those of terrestrial motion sickness, a common aetiology between the two may be suspected. However, there is a poor correlation between susceptibility of the two forms; crew members prone to motion sickness on earth were not necessarily always sufferers of space motion sickness, and vice versa (Davis *et al.*, 1988).

1.1.2.3 Adaptation of the pulmonary system

Prolonged exposure to a pressurised, artificial atmosphere, such as that experienced during space flight or in the International Space Station, coupled with a microgravity environment may be expected to affect pulmonary function to some degree. Indeed, changes in ventilation, perfusion, functional residual capacity and residual volume occur within hours of exposure to microgravity (Prisk, 2000). Perhaps surprisingly, and despite these changes, lung function has not been found to be seriously impaired during or following even longer duration spaceflight. On the contrary, some aspects of pulmonary function show improvement; increased

homogeneity of tissue ventilation and perfusion was associated with improved membrane diffusion capacity during nine days of microgravity (Verbanck *et al.*, 1997). This improvement may be countered somewhat by decreases in functional residual capacity and forced vital capacities (Elliott *et al.*, 1996; Prisk *et al.*, 1994), although the overall effect of these changes is considered negligible (West *et al.*, 1997).

1.1.2.4 Adaptation of the immune system

The thorough health screening and monitoring that precedes an astronaut's inclusion in a space program minimises to a large extent the possibility of infection with pathogenic microorganisms during the mission (Taylor *et al.*, 1986). Nonetheless, infectious disease has occurred during spaceflights, including gingivitis and skin infections (Ferguson *et al.*, 1975; Taylor & Nicogossian, 1977). These types of host-microbe infections suggest that immune function may be reduced sufficiently by microgravity that the tight regulation of the normally benign microflora of crew members is impaired (Hawkins *et al.*, 1975).

Extensive investigation into the effect of microgravity on the immune system has confirmed an overall suppression in both primary and secondary immune responses (Manie *et al.*, 1991; Sonnenfeld, 1999). Delayed-type hypersensitivity skin responses to several common antigens were reduced following spaceflight durations as short as 3 – 5 days (Taylor & Janney, 1992). Analysis of leukocyte populations following spaceflight reveals several changes, including a reduction in the number of eosinophils and lymphocytes. In addition to decreased numbers, the activity of lymphocytes was also found to be decreased in astronauts compared to pre-flight values (Taylor & Dardano, 1983).

1.2 CARDIOVASCULAR ADAPTATION TO MICROGRAVITY

Cardiovascular adaptation to microgravity, often referred to as *cardiovascular deconditioning*, has been observed in astronauts after space flights as short as 24 hours (Charles *et al.*, 1994; Ferguson *et al.*, 1975; Johnston & Dietlein, 1977; Watenpaugh & Hargens, 1996). Such adaptation is manifested primarily by orthostatic intolerance – the inability to maintain adequate cerebral perfusion upon standing when the astronaut returns to a 1-G environment, as determined by the inability to complete a 10 min stand test (Buckey *et al.*, 1996; Johnston & Dietlein, 1977). The consequences of this phenomenon are obviously a hindrance to the post-flight debriefing process and can prolong the time required for full recovery from the mission. Furthermore, such adaptations following microgravity exposure mirror to a large degree the cardiovascular deconditioning following prolonged bed rest, such as following spinal cord injury, and may have relevance to individuals with chronic orthostatic intolerance (Convertino, 2002; Vaziri, 2003).

1.2.1 Observations from human studies of actual and simulated microgravity

With the obvious limitations to conducting controlled physiological experiments in space, coupled to the small number of space flights each year - to date less than 650 people have flown in space (Aubert *et al.*, 2005) - mechanisms underlying cardiovascular adaptation to microgravity remains poorly understood. Sufficient and accurate measurements of baseline physiology alone presents a significant challenge, given the temporal and spatial constraints associated with current space missions; scientific experimentation, investigating pharmacological or non-pharmacological countermeasures to such adaptations in human subjects is even more scarce. Despite these challenges, much research, including the SpaceLab missions of the 1980s and 1990s (specifically designed to investigate changes in human and

laboratory animal physiology in space) have provided excellent insights into the mechanisms underlying the physiological responses to microgravity, particularly in the early stages of space flight (Gabrielsen *et al.*, 1995; Johnston & Dietlein, 1977; Sulzman, 1996).

The similarities between many of the physiological responses to microgravity and prolonged bed rest resulted in the development of the head-down bed rest (HDBR) model for simulating microgravity on earth (Pavy-Le Traon *et al.*, 2007). Indeed, this modification of the original trial by Dietrich *et al*. (1948) involving a -6° head down tilt has proved an invaluable tool for simulating microgravity, with particular usefulness for reproducing the effects of spaceflight on the musculoskeletal and cardiovascular systems. In this section, the observations relating from both spaceflight and HDBR studies are discussed.

1.2.1.1 Microgravity causes central hypovolaemia

The predominant changes in renal function observed in response to microgravity exposure involve body fluid volume and electrolyte homeostasis (Charles & Lathers, 1991; Huntoon *et al.*, 1994). Reductions in total circulating blood volume have been observed in most studies that include such measurements (Johnson, 1979; Johnston & Dietlein, 1977; Leach *et al.*, 1976). The hypovolaemic response to microgravity, particularly in light of the expected central fluid shift, was predicted by physiologists long before the advent of space flight. However, early studies during space flight elicited somewhat surprising results. Indeed, total circulating blood volume was decreased, but the anticipated classical *Henry-Gauer* reflex (Henry *et al.*, 1956) - predicted based on observations in the ground-based 'head-down tilt' model - did not occur. Natriuresis not only failed to increase, but sodium excretion was less compared with pre-flight supine measurements (Drummer *et al.*, 1993; Norsk *et al.*, 1995) and urine production has also been reported to be attenuated (Drummer *et al.*, 2000; Johnston & Dietlein, 1977; Norsk *et al.*, 2000). Proposed mechanisms for this anomalous response include attenuation of the

expected rise in atrial pressure (and therefore less of an increase in *atrial natriuretic peptide* secretion compared to what may be predicted) due to increased distensibility of the pulmonary vasculature produced by decreased pleural pressure (Videbaek & Norsk, 1997). Instead of diuresis/natriuresis, chronically increased extravasation of fluid into the interstitium and reduced erythrocyte volume has been suggested as the primary cause of decreased circulating blood volume (Alfrey *et al.*, 1996; Leach *et al.*, 1996).

As might be expected in response to hypovolaemia, plasma antidiuretic hormone (ADH) levels are increased between 5 and 24 hours after exposure to microgravity, and remain more than two-fold higher than pre-flight levels until at least 5 days (Leach *et al.*, 1976). As hypovolaemia persists for the duration of microgravity exposure, the normal physiological effect of increasing ADH is presumably negated by the several other changes to endocrine function observed in microgravity (Drummer *et al.*, 2000). Plasma renin activity has been observed to fluctuate greatly during the time course of the mission: an initial decrease occurs within hours of launch, followed by an increase compared to pre-flight values approximately 48 hrs later (Leach, 1986; Leach *et al.*, 1996). Furthermore, aldosterone levels are also increased, likely in an attempt (together with increased ADH release) to restore blood volume (Drummer *et al.*, 2001).

1.2.1.2 Microgravity produces a cephalad fluid shift

As mentioned previously, probably the most pronounced and immediate cardiovascular change upon entering microgravity is the translocation of blood from the lower extremities to the thoracic and cephalic circulation (Figure 1-2), manifested primarily by reduced leg volume and facial oedema (Charles & Lathers, 1991; Christensen *et al.*, 2001; Johnston & Dietlein, 1977). Coupled with an acute fluid shift from the interstitial space to the blood compartment in the lower body, this leads to transient rises in thoracic volume, cardiac output and arterial pressure

(Johnston & Dietlein, 1977). A more sustained increase in transmural pressure (calculated from measured cerebral blood flow velocity) in cerebral arteries has been reported (Kawai *et al.*, 1993), and has been proposed as the stimulus for the differential adaptation observed in cerebral arteries compared with peripheral (located outside of the brain, heart and other major organs) arteries from animal models of simulated microgravity (Wilkerson *et al.*, 1999; Zhang *et al.*, 2001).

Despite the observed reductions in circulating blood volume, many studies have revealed that hypovolaemia may play only a peripheral role in the orthostatic intolerance observed after microgravity exposure, as maintenance or restoration of blood volume has not been found to be a consistently effective countermeasure (Buckey *et al.*, 1996; Convertino, 2005; Meck *et al.*, 2001). This has shifted emphasis away from the theory of hypovolaemia as the sole mechanism underlying cardiovascular deconditioning to the proposal that attenuated neurohumoral control of blood pressure (with particular emphasis on inadequate peripheral artery vasoconstriction), coupled with inappropriate cerebral artery autoregulatory vasoconstriction, as co-determinants of the inability to maintain adequate cerebral perfusion during orthostatic challenge (Blomqvist *et al.*, 1994; Buckey *et al.*, 1996; Herault *et al.*, 2000; Mulvagh *et al.*, 1991; Zhang *et al.*, 1997).

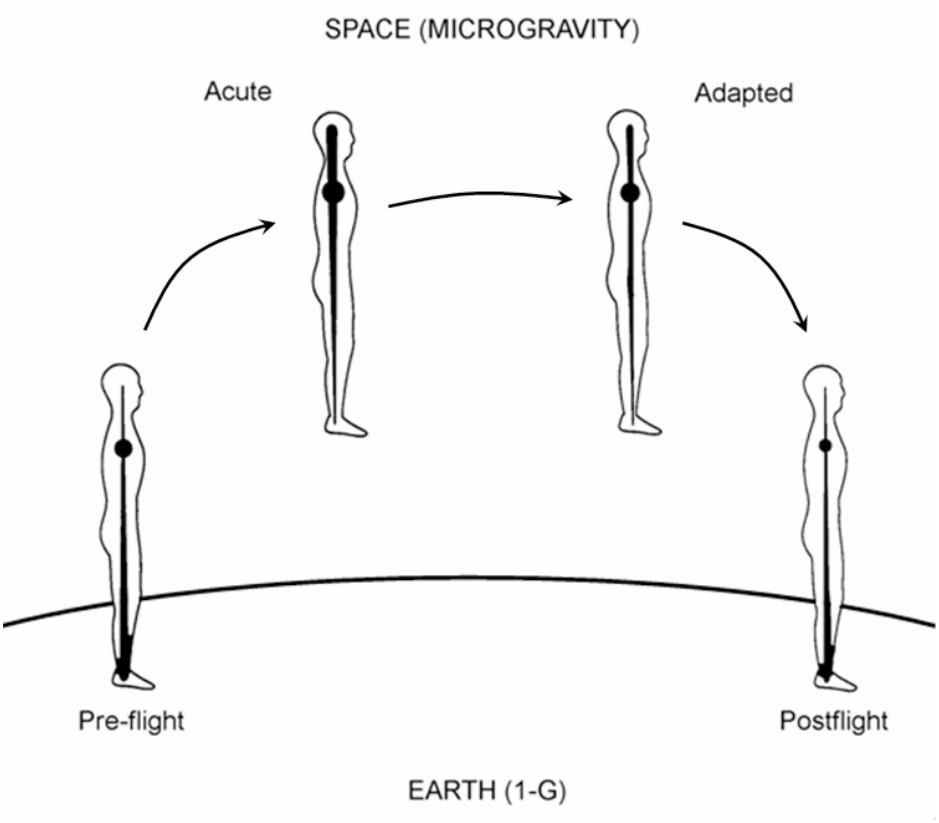

Figure 1-2 - Diagrammatic representation of body fluid distribution in response to microgravity
Acute exposure to microgravity results in a significant cephalad fluid shift; the effects of which result in readily-observable symptoms, including periorbital puffiness, facial oedema, distension of the veins in the temple and forehead, and nasal congestion. These symptoms are accompanied by decreases in lower limb volume, with decreases in calf circumference of up 30% reported within 48 hrs of spaceflight. Within 3-5 days, adaptation of the cardiovascular system compensates for the cephalad fluid shift, in addition to the decreases observed in plasma volume; overall, these adaptations result in compromised ability to maintain adequate cardiac output, total peripheral resistance and cerebral perfusion during orthostatic challenge, i.e. orthostatic intolerance (adapted with kind permission from Charles *et al.*, 1994).

1.2.1.3 Microgravity affects autonomic control of blood pressure

Due to the obvious role of autonomic baroreceptor-mediated control of arterial blood pressure in response to orthostatic challenge, it is not surprising that dysfunction of this process

has received much attention as a possible mechanism of cardiovascular deconditioning and the associated orthostatic intolerance (Convertino *et al.*, 1990; Crandall *et al.*, 1994). Unfortunately, research in this area has proved to be disappointingly inconclusive, plagued by conflicting results and a high degree of variability between studies, even from the same laboratory during different missions (Convertino, 2002; Watenpaugh & Hargens, 1996). One consistent observation is sustained bradycardia during space flight compared with pre-flight (Gundel *et al.*, 2002; Migeotte *et al.*, 2003); other findings also support a theory of vagal predominance in response to microgravity (Aubert *et al.*, 2005; Beckers *et al.*, 2003).

A reduction in stroke volume has been observed repeatedly in response to orthostatic challenge following either spaceflight or HDBR (Buckey *et al.*, 1996; Bungo & Johnson, 1983; Convertino *et al.*, 1994; Whitson *et al.*, 1995). Given the central role that stroke volume plays in determining cardiac output (and therefore arterial pressure), it is reasonable to assume that any changes in the key determinants of stroke volume (i.e. preload, afterload, myocardial function and autonomic cardiac control)(Rowell, 1993) in response to orthostatic stress would have substantial effects on the ability to maintain adequate cardiac output and arterial pressure. For this reason, many spaceflight and HDBR studies have included measurements of indices of the above determinants (Buckey *et al.*, 1996; Bungo & Johnson, 1983; Convertino *et al.*, 1994; Whitson *et al.*, 1995). Perhaps surprisingly, significant changes in intrinsic cardiac function have not been observed in response to microgravity (Bungo & Johnson, 1983; Watenpaugh & Hargens, 1996), and while some slight changes in myocardial mass have been demonstrated in longer duration missions, this is thought to reflect negative caloric balance and general loss of body mass routinely observed, and is not associated with deleterious effects on cardiac function (Bungo & Johnson, 1983; Perhonen *et al.*, 2001). In addition to changes in vagal activity, some evidence exists suggesting cardiac sympathetic neural traffic is altered following spaceflight or

HDBR, but as this was not correlated with significant effects on overall cardiac function, the relevance of these finding to the effects of microgravity on the control of arterial pressure is unclear (Convertino, 2002; Fritsch-Yelle *et al.*, 1994; Whitson *et al.*, 1995).

As proposed by Blomqvist and colleagues (Blomqvist *et al.*, 1994; Buckey *et al.*, 1993) it is fair to anticipate that adaptation of the autonomic/neurohumoral control of arterial pressure may occur at many levels, including afferent neuronal input (Grigoriev & Egorov, 1996; Kirsch *et al.*, 1984), central processing (Blomqvist *et al.*, 1994; Fritsch-Yelle *et al.*, 1994) efferent neuronal output (Cooke *et al.*, 2000; Eckberg, 2003) and the effector organ i.e. the vasculature (Delp *et al.*, 2000; Zhang, 2000). While investigation of each component is certainly warranted, the scope of this thesis is limited to how adaptations of the vasculature in response to microgravity affect its function in relation to maintenance of adequate arterial blood pressure and cerebral perfusion.

1.2.1.4 Microgravity increases venous compliance

From the observations discussed earlier regarding changes in stroke volume in response to spaceflight or HDBR, reductions in cardiac preload (i.e. cardiac filling pressure) would have obvious negative implications for maintenance of cardiac output in response to orthostatic challenge. Indeed, increased venous compliance, defined as the ratio of a change in volume to a simultaneous change in transmural pressure (Shoukas & Sagawa, 1971), has been demonstrated following both spaceflight and HDBR (Belin de Chantemele *et al.*, 2004). Given that ~70 % of total blood volume is contained within the venous system, changes in its capacity have significant effects on venous return, and subsequently, cardiac output and blood pressure (Rowell, 1993). For this reason, together with blood volume, arterial compliance/resistance, heart rate and contractility, venous tone plays a central role in the regulation of blood pressure (Greenway, 1981; Guyton *et al.*, 1964).

Early increases in venous compliance upon initial exposure to microgravity are likely due to reduced leg interstitial fluid volume and pressure (Kirsch *et al.*, 1993; Nixon *et al.*, 1979), whereas following prolonged exposure to microgravity, adaptations (reduction) of skeletal and vascular muscle mass may increasingly contribute to reductions in compliance (Arbeille *et al.*, 2008; Hoffler *et al.*, 1975; Thornton *et al.*, 1977). These findings are limited by the techniques used to determine compliance - measurement of compliance using conventional venous occlusion plethysmograpy does not allow differentiation between the effects of extracellular vs. vascular components - therefore the relative contributions of each are unclear (Belin de Chantemele *et al.*, 2004; Watenpaugh & Hargens, 1996). However, observations from leg blood flow studies following spaceflight suggest that sympathetically-mediated venoconstriction occurs (presumably to oppose the increases in compliance). These findings have led to the consensus that decreased skeletal muscle mass and fluid pressure, and not changes in venous tone, are largely responsible for the increases in leg compliance (Arbeille *et al.*, 2008; Buckey *et al.*, 1988; Convertino *et al.*, 1988; Thornton *et al.*, 1977; Watenpaugh & Hargens, 1996).

1.2.1.5 Microgravity compromises the ability to raise total peripheral resistance

There is an increasing body of evidence suggesting that adaptation at the level of vascular structure and function plays a major role in cardiovascular deconditioning (Aubert *et al.*, 2005; Watenpaugh & Hargens, 1996). It has been long established that vasoconstriction of peripheral arteries is essential for maintaining adequate arterial pressure upon orthostatic challenge (Rowell, 1993; Watenpaugh & Hargens, 1996). Investigation of structural changes in the vasculature in response to microgravity in humans is obviously less feasible than in animal models, so the vast majority of such research has been performed using animal models. However, much functional data exists to support the theory of altered vascular function following spaceflight or HDBR. Impairment in the ability to increase total peripheral resistance

(TPR) in response to orthostatic challenge following spaceflight has been observed repeatedly (Waters *et al.*, 2002). Interestingly, this has been shown to occur despite increases in circulating plasma catecholamine concentrations (Whitson *et al.*, 1995), although results from a similar study suggest that a simple attenuation of the pressor response to catecholamines may not always explain such findings (Fritsch-Yelle *et al.*, 1996b).

Two main hypotheses have emerged based on the above (and other) observations regarding the impaired ability of the sympathetic nervous system to adequately elevate TPR following adaptation to microgravity: Convertino suggests that a reduced *vasoconstrictor reserve* is responsible, based on the finding that basal vasoconstriction was actually increased during supine rest following space flight or HDBR (Convertino, 2002; Gabrielsen *et al.*, 1995). This has been proposed to compromise the capacity to buffer hypotension upon orthostatic challenge. Moreover, while plasma volume, stoke volume, and baroflex function have all been observed to be decreased following exposure to real or simulated microgravity, negligible differences between astronauts with retained or compromised orthostatic tolerance have been found (Convertino, 2002). In contrast, a lower vasoconstrictor reserve has been repeatedly found in orthostatic intolerant subjects compared with tolerant subjects. This variable has been correlated to failure to complete a ten-minute stand test following space flight (Fritsch-Yelle *et al.*, 1996a; Fritsch-Yelle *et al.*, 1996b; Waters *et al.*, 2002), and furthermore, has been demonstrated as being a contributing factor to individual variability in orthostatic intolerance in healthy volunteers (Fu *et al.*, 2004a).

The alternative theory offered by Vaziri is that impaired vasoconstriction following microgravity exposure may be due to the chronic overproduction of endogenous vasoconstrictors such as noradrenaline and angiotensin II (Vaziri, 2003). The rationale being that overproduction of such mediators during exposure to microgravity would likely lead to

downregulation in receptor expression/signal transduction pathways and subsequently a decreased response to exogenous administration of the agonists (Vaziri, 2003). Indeed, the pressor response to vasoconstrictors such as noradrenaline has been consistently found to be blunted following exposure to microgravity or HDBR (Fritsch-Yelle *et al.*, 1996b; Hargens *et al.*, 1992; Whitson *et al.*, 1995).

A further hypothesis is that instead of diminished vasoconstrictor capacity, microgravity exposure alters vasodilator tone (Aubert *et al.*, 2005; Convertino *et al.*, 1997; Sandler *et al.*, 1985; Zhang, 2001). There is limited evidence supporting this hypothesis in human studies, with the primary rationale stemming from the observation that β-adrenoceptor-mediated vasodilatation was increased following 14 days HDBR (Convertino *et al.*, 1997). However, only a limited benefit was demonstrated in an earlier study investigating the effectiveness of propranolol as a countermeasure to the cardiovascular deconditioning following HDBR; in some cases, orthostatic intolerance was in fact exacerbated from excessive inhibitory chronotropic and inotropic effects (Sandler *et al.*, 1985). This hypothesis is also in contrast to findings from HDBR studies measuring forearm blood flow in response to various physiological challenges that suggest that vasodilatation is decreased following exposure to simulated microgravity (Demiot *et al.*, 2007; Shoemaker *et al.*, 1998). Interestingly, in a similar HDBR study, administration of a low calorie, low fat diet was found to offer some degree of protection against such adaptations (Hesse *et al.*, 2005).

1.2.1.6 Microgravity differentially affects cephalic and caudal arterial beds

In contrast to the aforementioned observations with regards to the peripheral vasculature (i.e. the vasculature outside of the brain, heart or other major organs), cephalic arteries appear to adapt differently to microgravity (Watenpaugh & Hargens, 1996; Wilkerson *et al.*, 2005; Zhang *et al.*, 2001). It has been proposed that exposure to microgravity produces a reversal of the

transmural pressures exerted on different vascular beds, relative to the *hydrostatic indifference point* (Hargens *et al.*, 1992). The hydrostatic indifference point is defined as the point in the circulatory system where hydrostatic pressure remains constant regardless of changes in body position relative to the gravitational vector (Gauer & Thron, 1965). In humans, this is estimated at being approximately at the level of the diaphragm. Under normal (1-G) conditions the cerebral vasculature experiences transmural pressures significantly lower than vascular beds caudal to the hydrostatic indifference point (Gauer & Thron, 1965), whereas upon exposure to microgravity removal of the hydrostatic pressure gradient results in equilibration of arterial pressures throughout the vasculature, resulting in increased and decreased transmural pressure in cephalic and peripheral vascular beds, respectively (Blomqvist *et al.*, 1983; Watenpaugh & Hargens, 1996). In order to maintain constant cerebral perfusion, the autoregulatory capacity of the cerebral vasculature alters resistance in response to changes in perfusion pressure, such that increased pressure (as observed during exposure to microgravity) results in increased vascular resistance (autoregulatory vasoconstriction), while decreases in pressure are compensated for by decreases in vascular resistance (autoregulatory vasodilatation)(Heistad *et al.*, 1983; Rowell, 1993). Autoregulation of cerebral arteries is discussed in further detail in a later section.

Using different techniques to measure cerebral blood flow/tissue perfusion at varying arterial pressures (and therefore estimate cerebral vascular resistance), exaggerated cerebral artery autoregulatory vasoconstriction/lack of autoregulatory vasodilatation - manifested by increased cerebral vascular resistance - has been suggested following space flight and bed rest (Arbeille *et al.*, 1996; Gazenko *et al.*, 1981; Herault *et al.*, 2000; Zhang *et al.*, 1997). Upon return to earth, the adaptation observed in cerebral artery function persisted for longer (up to 5 weeks) than most other cardiovascular adaptations reported to date (Gazenko *et al.*, 1981; Herault *et al.*, 2000). Furthermore, cerebral autoregulation over the lower end of the flow-

pressure curve was found to be impaired following HDBR (Zhang *et al.*, 1997). This has led to the suggestion that adaptation of the cerebral circulation to chronic elevation in perfusion pressure experienced during microgravity may contribute to postflight orthostatic intolerance (Watenpaugh & Hargens, 1996; Zhang, 2001).

Comparison of arterial control of blood flow between cerebral arteries and femoral arteries using echo-Doppler flowmetry following spaceflight has revealed a tendency towards decreased resistance in femoral arteries, thereby further decreasing the cerebral:femoral blood flow ratio (Herault *et al.*, 2000; Tobal *et al.*, 2001). Moreover, decreases in the cerebral:femoral blood flow ratio were associated with reduced orthostatic tolerance, supporting the growing consensus that cerebral vasculature adapts in the opposite way to vascular beds caudal to the hydrostatic indifference point (Purdy *et al.*, 2003; Tobal *et al.*, 2001). Together with the findings from animal models (discussed below), these observations have lead to the proposal of *differential adaptation* of the arterial system in relation to the hydrostatic indifference point as being a mechanism of orthostatic intolerance following exposure to microgravity (Purdy *et al.*, 2003; Zhang, 2001).

1.2.1.7 Insight from studies with potential countermeasures

In addition to the various physiological measurements made during and/or following exposure to microgravity, much has been learnt from the investigation of countermeasures prompted by these findings. The countermeasures investigated to date may be broadly divided into either pharmacological or non-pharmacological.

The use of currently available drugs to prevent orthostatic intolerance following spaceflight has resulted in limited success. Based on the initial observations of hypovolaemia following spaceflight and HDBR, expansion of plasma volume appeared an obvious and attractive countermeasure. Early studies involving acute oral saline loading immediately prior

to returning to earth showed modest effectiveness in preventing orthostatic intolerance following short (< 7 day) duration spaceflight (Bungo *et al.*, 1985). However, no benefit from this approach has been demonstrated for longer (> 7 day) missions, with no difference in upright stroke volume observed between control and saline-loaded crewmembers (Buckey *et al.*, 1996; Vernikos & Convertino, 1994).

The ineffectiveness of saline loading alone prompted the investigation of a more aggressive approach to plasma volume expansion. The mineralocorticoid fludrocortisone has been successfully used clinically to treat patients with orthostatic hypotension associated with hypovolaemia (Robertson & Davis, 1995), and therefore was investigated to determine its usefulness as countermeasure against microgravity-induced orthostatic intolerance. Two studies using the HDBR model demonstrated success with fludrocortisone in maintaining plasma volume and protecting against orthostatic intolerance, compared with control subjects receiving saline alone (Vernikos & Convertino, 1994; Vernikos *et al.*, 1991). Unfortunately, the effectiveness of fludrocortisone observed in HDBR studies was not reproduced when tested on astronauts returning to earth; despite restoration of plasma volume, no protection against orthostatic intolerance was demonstrated in subjects that received fludrocortisone compared with placebo-treated controls (Shi *et al.*, 2004). The findings from this study resulted in fludrocortisone being discontinued as a countermeasure for orthostatic intolerance following spaceflight, and provided researchers with further evidence that hypovolaemia alone may not be the predominant mechanism underlying the phenomenon.

As mentioned above, the hypothesis that β-adrenoceptor-mediated vasodilatation in response to circulating catecholamines was increased following microgravity led to the non-selective β-adrenoceptor antagonist propranolol being investigated as a countermeasure following HDBR (Sandler *et al.*, 1985). While some peripheral vasoconstriction was observed,

any potential benefits were outweighed by negative chronotropic and inotropic effects that actually reduced orthostatic tolerance. Instead of inhibiting β-adrenoceptor-mediated vasodilatation, increasing venous tone, venous return and peripheral vasoconstriction through the stimulation of smooth muscle α-adrenoceptors have been investigated with greater success.

The selective α_1-adrenoceptor agonist midodrine has shown some promise as a pharmacological countermeasure, showing protection against orthostatic intolerance when administered at the end of a 16 day HDBR period (Ramsdell *et al.*, 2001). These early findings have unfortunately been negated by the occurrence of an unwanted pharmacological interaction of midodrine with the antihistamine promethazine (commonly prescribed to astronauts for the treatment of space motion sickness), resulting in akathisia and associated aggressive behaviour (Platts *et al.*, 2006). For this reason, NASA has banned the use of midodrine in combination with promethazine.

Non-pharmacological countermeasures have been investigated with slightly more success than the pharmacological approach, and include various exercise regimes, exposure to lower body negative pressure (LBNP) and exposure to artificial gravity (Convertino, 2002; Purdy *et al.*, 2003). The latter may be achieved through the use of a short-arm human centrifuge, and ground-based studies have demonstrated some protection against orthostatic intolerance following HDBR, associated with increases in central blood volume, cardiac output, vasoconstrictor reserve and responsiveness to α-adrenoceptor stimulation (Clement & Pavy-Le Traon, 2004; Convertino, 1998; Zhang *et al.*, 2003). Unfortunately the use of artificial gravity as a countermeasure is problematic, as repeated exposure over the duration of the mission is required, taking up substantial amounts of crewmembers' productive time. In addition, the size and weight of even the smaller human centrifuges are prohibitively expensive; the estimated cost of taking 1 kg of material into space is approximately US$30,000 (Clement & Pavy-Le

Traon, 2004; Frey, 1996).

An alternative way of exposing subjects to orthostatic stress in an attempt to counter the cephalad fluid shift in response to microgravity is through the use of LBNP. This method has demonstrated some benefit in HDBR studies with short daily periods of LBNP exposure affording some protection against orthostatic intolerance (Sun *et al.*, 2002). The beneficial effects were not associated with restoration of plasma volume, and unfortunately LBNP was not found to be effective in preventing orthostatic intolerance in a separate HDBR study or following spaceflight (Pavy-Le Traon *et al.*, 1995; Sawin *et al.*, 1998). While LBNP alone may lack effectiveness in the prevention of orthostatic intolerance following exposure to actual or simulated microgravity, its combination with exercise has shown potential as a useful countermeasure (Convertino, 2002; Purdy *et al.*, 2003). When evaluated alone, bouts of acute maximal exercise were found to restore blood volume and baroreflex function following HDBR and spaceflight, but only afforded marginal improvements in orthostatic tolerance (di Prampero & Antonutto, 1997; Engelke *et al.*, 1995; Greenleaf *et al.*, 1989). A series of studies have evaluated different combinations of LBNP and acute maximal exercise (Hargens *et al.*, 1991; Murthy *et al.*, 1994; Watenpaugh *et al.*, 2000; Watenpaugh *et al.*, 1994), with the overall findings resulting in the proposal of brief (40 min/day) periods of intense exercise while under LBNP followed by 5 min of LBNP alone as the most effective combination in improving orthostatic tolerance following HDBR (Watenpaugh *et al.*, 2007).

While the most recent studies of non-pharmacological countermeasures appear to offer more time-efficient solutions to the problem of post-flight orthostatic intolerance, the time, personnel and spatial resources such approaches demand are less than ideal. From the insights gained from both pharmacological and non-pharmacological countermeasures investigated to date, further understanding of the mechanisms underlying cardiovascular deconditioning in

response to microgravity are required if wholly-successful countermeasure are to be developed.

1.2.2 Observations from animal models of simulated microgravity

1.2.2.1 Development of the head down tilt (HDT) rodent model

The development of the HDBR model as an earth-based simulation of microgravity has allowed significant insight into potential mechanisms underlying cardiovascular deconditioning, in addition to adaptation of other physiological systems, such as muscle atrophy and bone demineralisation in the musculoskeletal system (Pavy-Le Traon *et al.*, 2007). As with all research into human conditions or diseases, ethical considerations limit the number and types of experiments that may be conducted on human subjects. For this reason, appropriate animal models are essential for advancing our understanding of how the cardiovascular system adapts, particularly with regards to assessing changes in structure/function of different vascular beds (Zhang *et al.*, 2001). Furthermore, knowledge from animal models allows the planning and design of more productive and efficient human studies (Tkacs & Thompson, 2006).

Several species have been used for this purpose, including mice (Powers & Bernstein, 2004) and non-human primates (Koenig *et al.*, 1998), however for cardiovascular studies the head down tilt (HDT) rat model has become the most widely used and accepted animal model, despite the obvious limitations of using a quadruped to simulate a condition experienced by a bipedal species such as humans (Morey-Holton & Globus, 2002). Such limitations primarily relate to the absence of a substantial hydrostatic pressure gradient along the cephalocaudal axis in quadrupeds compared with an erect species such as man. The various neural mechanisms involved in the normal human physiological response to standing, including sympathetic stimulation of the arterial and venous vasculature (to increase TPR and cardiac filling pressure, respectively) and increased sympathetic stimulation of the sinoatrial node (to increase heart rate

and therefore cardiac output) play less of a role in the normal physiology of quadrupeds, utilised only during brief periods of rearing onto hindlegs (Morey *et al.*, 1979; Rowell, 1993).

Initially developed by Morey and co-workers at the NASA Ames Research Centre (California, USA) in an attempt to reproduce musculoskeletal adaptation to microgravity, the HDT model was originally referred to as the hind limb unweighting (HLU) model, reflecting the significance of unloading the hind limbs to simulate microgravity conditions (Morey *et al.*, 1979). For this reason, the majority of the literature uses the HLU terminology when citing this model, even when the musculoskeletal system may not be the focus of research. More recently researchers using this model for investigating cardiovascular deconditioning following microgravity have favoured the HDT designation, indicating the importance of a cephalad fluid shift - as in the HDBR human analogue – in the aetiology of such adaptations.

Aside from the practical advantages over smaller animals (mice) or larger animals (rabbits, monkeys), the cardiovascular adaptation exhibited by HDT rats mimics to a large extent that observed in human microgravity or HDBR studies, including cephalad fluid shifts, transient increase in central venous pressure, vasoconstrictor hyporesponsiveness, hypotension in response to orthostatic challenge and an impaired increase in TPR in response to orthostatic challenge (Hargens *et al.*, 1984; Martel *et al.*, 1996; McDonald *et al.*, 1992; Morey *et al.*, 1979; Morey-Holton & Globus, 2002; Overton & Tipton, 1990; Wilkerson *et al.*, 2005; Woodman *et al.*, 1995). Table 1-1 compares some of the main adaptations observed following actual or simulated microgravity between spaceflight and ground-based models.

Table 1-1 - Comparison of major physiological adaptations to actual and simulated microgravity

	spaceflight	*HDBR*	*HDT*
Bone density	↓	↓	↓
Muscle mass	↓	↓	↓
Neurovestibular disturbances	✓	x	?
Blood volume	↓	↓	↓
Cardiac output	↓	↓	↓
Baroreflex function	↓	↓	↓
Ability to raise TPR	↓	↓	↓
Differential arterial adaptation	?	?	✓
Orthostatic tolerance*	↓	↓	↓

* - Defined as resistance to the development of presyncopal symptoms upon exposure to orthostatic stress in human studies, or the incidence/severity of hypotension/tachycardia/decreased cerebral perfusion in animal studies; ↓ - decrease; ✓ - adaptation observed; x – adaptation not observed; ? – unknown if adaptation occurs; HDBR – head down bed rest; HDT – head down tilt; TPR – total peripheral resistance. (data compiled from Convertino, 2002; Morey-Holton *et al.*, 2002; Nicogossian, 1994; Pavy-Le Traon *et al.*, 2007; Zhang, 2001).

1.2.2.2 Adaptation of the venous system

Together with hypovolaemia, increased peripheral venous compliance has been suggested as a contributing factor to attenuated cardiac filling pressure and subsequent decreases in stroke volume and cardiac output following exposure to actual or simulated microgravity (Buckey *et al.*, 1988; Convertino *et al.*, 1988; Dunbar *et al.*, 2001). As with human studies into the effect of microgravity on venous function, few studies using animal models have investigated such adaptation to date. Using the pressure-diameter relationships in isolated small

mesenteric veins as an index of vascular capacitance, Dunbar and colleague suggested that unstressed vascular volume (approximately two-thirds of the total blood volume that fills the circulatory system to maximum capacity without increasing transmural pressure)(Rothe, 1983) increases following exposure to simulated microgravity, and subsequently may not be adequately decreased upon sympathetic stimulation to maintain cardiac output during orthostatic challenge (Dunbar *et al.*, 2000). Furthermore, the sensitivity of isolated strips of vena cava to noradrenaline has also been found to be decreased following HDT (Sayet *et al.*, 1995). Vascular capacitance function has also been measured *in vivo* in an elegant study utilising a constant flow, constant right atrial pressure cardiopulmonary bypass technique (Dunbar *et al.*, 2001). Total systemic vascular compliance and venous compliance were found to be increased in rats following simulated microgravity, and were proposed as being potential mechanisms for the aforementioned decreased in cardiac filling pressure following exposure to microgravity.

The effects of microgravity on the venous system are incompletely understood. While alterations in venous function have been observed in animal models, current findings from human studies suggest that adaptations in venous function may exacerbate the observed decreases in orthostatic tolerance observed after spaceflight, but may not play a predominant role in its aetiology (Convertino, 2002; Watenpaugh & Hargens, 1996; Zhang, 2001).

1.2.2.3 Structural adaptation of conduit and resistance arteries

The most consistent finding regarding structural changes following simulated microgravity is increased cross-sectional area (CSA) of the tunica media (Chew & Segal, 1997; Mao *et al.*, 1999; Wilkerson *et al.*, 1999). The extent of these changes in relation to anatomical location appears somewhat complex however; after 10-14 weeks of HDT increased medial CSA was observed in carotid, axillary, iliac and femoral arteries (Chew & Segal, 1997). During shorter periods (4 weeks) of HDT, an apparent opposing structural adaptation between cephalic

and caudal arteries has been observed, with decreases in medial CSA measured in the femoral and anterior tibial arteries and increases in common carotid and basilar arteries (Mao *et al.*, 1999). In contrast, from investigating the effects of two weeks of simulated microgravity on passive and active mechanics of isolated rat aortas, Papadopoulos *et al.* demonstrated that contractile deficits in arteries from HDT rats were not associated with any changes in medial CSA, or differences in either passive or active Cauchy stress-stretch responses (Papadopoulos & Delp, 2003).

Regional differences have also been observed following 2 weeks of HDT, which resulted in an increase in basilar artery medial CSA but no changes in this variable in either the mesenteric and splenic arteries (Wilkerson *et al.*, 1999). The mechanical properties of isolated carotid and femoral arteries following 20 days of HDT have also been investigated (Hwang *et al.*, 2007). In this study, decreases in expression of myosin light chain-20 in both arteries were not associated with any changes in active length-force relationships. However in femoral (but not carotid) arteries, decreases in myosin heavy chain protein expression levels were associated in decreased contractile response to KCl. In carotid arteries, decreases in vessel wall compliance were determined from a steeper length-passive force curve in HDT rats compared with control.

A feasible explanation for the regional differences in arterial morphological changes may be related to the reversal in transmural pressure exerted under microgravity conditions (described above). Cephalic arteries experience increased transmural pressure, stimulating smooth muscle cell hyperplasia/hypertrophy while caudal arteries are subject to lower than normal transmural pressure (thus removing any basal proliferative stimuli) leading to differential adaptation (Watenpaugh & Hargens, 1996; Zhang, 2001). In addition to changes in transmural pressure, changes in shear stress as a result of changes in blood flow have also been

proposed as a stimulus for structural adaptation of arteries to microgravity (Delp *et al.*, 2000). The lack of structural adaptation observed in the mesenteric/splenic arteries may reflect a smaller distance from the hydrostatic indifference point compared to arteries that exhibit marked changes (Zhang, 2001). The concept of differential adaptation may also be observed in measurements of blood flow to different regions of the skeletal system (Colleran *et al.*, 2000). In this study it was demonstrated that following 28 days of HDT in rats, tibial and femoral bone blood flow was decreased, while flow to the mandible, clavicle and humerus was increased.

Alterations in arterial function as a result of vascular remodelling have also been investigated in rat skeletal muscle arterioles following 2 weeks of HDT (Delp, 1999; Delp *et al.*, 2000). The nature of the structural adaptation of hind limb skeletal muscle arterioles was found to vary between muscles composed primarily of type I (slow twitch) and type IIb (very fast twitch) muscle fibres (Delp *et al.*, 2000). Decreases in medial CSA were observed in both the soleus (slow twitch) and the superficial white portion of the gastrocnemius (very fast twitch) muscles, however the decrease was associated with a reduction in vessel diameter in the soleus compared with a reduced medial thickness in the gastrocnemius. Furthermore, endothelial-dependent vasodilatation was impaired in arterioles from soleus, but not gastrocnemius muscles leading to the author's suggestion that perhaps reduced blood flow and wall shear stress are stimuli for the observed adaptation in slow twitch muscle arterioles whereas gastrocnemius arterioles structurally adapt in response to reductions in transmural pressure. In a separate study (Delp, 1999), diminished myogenic autoregulatory and contractile responsiveness to KCl/phenylephrine was observed in skeletal muscle arterioles from gastrocnemius muscle, but not soleus, following 2 weeks of HDT. These findings provide evidence for a potential mechanism by which adaptation of skeletal muscles arterioles may contribute to a compromised

ability to elevate total peripheral vascular resistance following exposure to simulated microgravity.

1.2.2.4 Functional adaptation: arterial vasoconstrictor tone.

The regional differences in vascular structural adaptation to microgravity also extend to functional alterations. Isolated abdominal aortic rings from 2 week HDT rats exhibited decreased maximal isometric contraction to both agonist- and KCl-induced contractions (Delp *et al.*, 1993). A more comprehensive study revealed vasoconstrictor responses in several hindquarter arteries were decreased following 2 and 4 week HDT, but basilar artery constrictor responses to the same stimuli were significantly enhanced, suggesting that differential functional adaptation occurs following HDT, as observed in human HDBR studies (Tobal *et al.*, 2001; Zhang, 2000; Zhang *et al.*, 2001).

Potential mechanisms for the observed changes in vasoconstrictor tone are unclear, and may not only reflect structural changes in the muscular composition of the tunica media of arteries; there is increasing evidence to suggest that the relative contribution of various vasoconstrictor mediators is altered following microgravity (Zhang, 2001). Sangha and colleagues observed increased noradrenaline-induced contraction of isolated carotid artery rings from HDT rats, and demonstrated that this may, in part, be mediated by upregulation of thromboxane A_2 (Sangha *et al.*, 2001). Enhanced contractile responses to 5-hydroxytrytamine have also been observed in isolated basilar arterial rings following HDT (Zhang *et al.*, 2001).

Corresponding *in vivo* studies corroborating these observations are not in abundance. As with most physiological/pharmacological research, *in vitro* isolated tissue experiments (whilst affording the researcher far greater control over the nature/composition of the external environment of the tissue, such as changes to Ca^{2+} concentration or extracellular drug addition) lack many of the neuro-humoural factors that may greatly affect aspects such as basal smooth

muscle tone produced by the sympathetic nervous system or circulating vasoconstrictor hormones (e.g. angiotensin II, vasopressin). Therefore, the findings from *in vitro* studies need ideally to be interpreted together with corresponding *in vivo* experiments. Unfortunately in the area of cardiovascular adaptation to simulated microgravity, such *in vivo* studies are relatively sparse.

A marked attenuation in the pressor response to noradrenaline has been demonstrated in anaesthetised rats following 20 days HDT (Sangha *et al.*, 2000). A reduced pressor response to sympathomimetics drugs was also observed in HDT rats by Overton and Tipton, who went on to report that this was associated with attenuated increases in resistance of the mesenteric vascular bed (Overton & Tipton, 1990). These findings are in agreement with later studies, including observations from space-flown rats (Hatton *et al.*, 2002). Impairment in the ability of the sympathetic nervous system to appropriately distribute cardiac output following 15 days of HDT was also attributed to decreased vascular responses to catecholamines (McDonald *et al.*, 1992). Interestingly, studies investigating regional flow have observed a new steady state of 60% of baseline values of hind limb blood flow after just 5 days of HDT (Roer & Dillaman, 1994; Woodman *et al.*, 2001).

Increases in cerebral vascular resistance have been demonstrated following periods of HDT ranging from 10 mins to 28 days (Wilkerson *et al.*, 2002). In this study Wilkerson and colleagues observed a reduction in cerebral blood flow by 48, 24, and 27% following 10 min and 7 and 28 days of HDT, respectively, associated with increases in total cerebral vascular resistance of 116, 44, and 38% following 10 min and 7 and 28 days of HDT, respectively, relative to that during control standing. In a separate study utilising radiolabeled microspheres, 21 out of 38 brain regions exhibited decreased basal blood flow following 2 weeks of HDT (Wilkerson *et al.*, 2005), perhaps reflecting increases in myogenic tone (Geary *et al.*, 1998).

Interestingly, increases in myogenic tone were observed in pressurised mesenteric arteries isolated from rats exposed to 28 days of HDT, while vasoconstrictor responses to catecholamines, serotonin and KCl were unchanged (Looft-Wilson & Gisolfi, 2000).

Vasoconstrictor responses to sympathetic nerve stimulation have also been investigated following simulated microgravity (De Salvatore *et al.*, 2004). In this study, perivascular nerve stimulation induced frequency-dependent increases in perfusion pressure of isolated perfused mesenteric vascular beds were blunted in preparations from 2 week HDT rats compared to control. Conversely, vasoconstrictor responses to noradrenaline added to the perfusate were potentiated. Vasodilator responses of noradrenaline-preconstricted mesenteric vascular beds to either perivascular nerve stimulation or isoprenaline were unchanged following simulated microgravity.

1.2.2.5 Functional adaptation: arterial vasodilator tone

Increased vasodilator tone, as opposed to attenuated vasoconstrictor tone has been proposed as a possible mechanism for the impaired pressor response and post-suspension hypotension observed in this model (Convertino, 2002; Zhang, 2001). Several studies have demonstrated changes in the vasodilator response to acetylcholine: decreases in acetylcholine-induced vasodilatation in isolated abdominal aortic rings and rat soleus feed arteries following HDT have been reported, suggested as being associated with changes in acetylcholine-mediated nitric oxide and prostacyclin (PGI_2) release (Delp *et al.*, 1995; Jasperse *et al.*, 1999; Schrage *et al.*, 2000). Furthermore, Eatman and colleagues suggested that the increased PGI_2 and nitric oxide production measured following 7 days of suspension may contribute to the decrease in MAP following resumption of normal horizontal posture observed in these animals (Eatman *et al.*, 2003a). This theory is supported somewhat by the observation that production of both nitric oxide and PGI_2 has been found to be increased in cultured human endothelial cells following

exposure to microgravity (Carlsson *et al.*, 2002). In contrast, inhibition of cyclo-oxygenase had no effect on endothelium-dependent vasodilatation in isolated middle cerebral arteries from HDT or control rats, suggesting that at least in this artery, PGI_2 plays a negligible role in the regulation of vascular tone (Prisby *et al.*, 2006).

As previously described, increased β_2-adrenoceptor-mediated vasodilatation has been observed in a ground-based simulation of microgravity (Convertino *et al.*, 1997), leading to the investigation of propranolol as a potential countermeasure (Sandler *et al.*, 1985). Somewhat surprisingly, very few studies into altered β-adrenoceptor function from animal models of simulated microgravity have been reported, perhaps reflecting the current convention of journals not readily publishing 'negative' results (i.e. studies that do not find a significant difference between experimental and control groups/conditions). The few studies that have investigated the effects of simulated microgravity on vascular β-adrenoceptor function have not revealed many substantial changes. Vasodilator responses to isoproterenol in first order arterioles isolated from rat soleus and gastrocnemius muscles were demonstrated to be unchanged following two weeks of simulated microgravity, while the vasodilation to adenosine was found to be decreased (McCurdy *et al.*, 2000). Furthermore, Fadiukova *et al.* found no significant difference in the vasodilator response to isoproterenol in either basilar or middle cerebral arteries following 14 day HDT (Fadiukova *et al.*, 2005). These findings were partially supported by a study by Ma et al. that demonstrated no change in the response to isoproterenol in isolated carotid, abdominal aortic and mesenteric arterial rings following 8 weeks of HDT, but did observe a decrease in the vasodilator response in femoral artery rings (Ma *et al.*, 1998b).

1.2.3 Potential role for changes in the L-arginine/nitric oxide pathway

As discussed above, an increasing body of evidence suggests the cephalad fluid shift induced by actual or simulated microgravity induces functional changes in both vasoconstrictor

and vasodilator mechanisms that regulate vascular tone. From initial observations regarding the changes in the vasodilator responses to acetylcholine following HDT, the altered contribution of endothelium-derived vasodilator mediators, including nitric oxide, PGI_2 and endothelium-derived hyperpolarising factor (EDHF) has been proposed as a possible mechanism underlying differential adaptation (and ultimately cardiovascular deconditioning) to microgravity (Prisby *et al.*, 2006; Wilkerson *et al.*, 2005; Zhang, 2001). Of the three mediators nitric oxide has received the majority of attention from researchers, and has been demonstrated as being differentially altered between different vascular beds following HDT (Purdy *et al.*, 2003; Zhang, 2001).

1.2.3.1 Role of L-arginine/nitric oxide pathway in control of vascular tone

The essential role played by nitric oxide in the regulation of vascular tone has been demonstrated on many levels since its discovery as an endothelium-derived relaxing factor in the 1980s (Ignarro *et al.*, 1987; Moncada & Higgs, 2006; Palmer *et al.*, 1987). Furthermore, alterations in the L-arginine/nitric oxide pathway have been implicated in several vascular disease states, including atherosclerosis, hypertension, pre-eclampsia, heart failure, and the cardiovascular complications associated with diabetes (Maxwell, 2002). Such alterations may occur at several points in the pathway leading from the biosynthesis of nitric oxide to the main molecular effector mechanism, activation of soluble guanylate cyclase. Therefore, in the context of adaptation of the L-arginine/nitric oxide pathway following exposure to microgravity, it is useful to briefly review the current understanding of this pathway (and its contribution to the tone of different vascular beds), and how it may be modulated physiologically and pharmacologically.

Biosynthesis of nitric oxide

Nitric oxide is synthesized from the amino acid L-arginine (together with cosubstrates

molecular oxygen and nicotinamide adenine dinucleotidephospate) by the *nitric oxide synthase* (NOS) family of enzymes in a two step process via the formation of *N*-hydroxyl L-arginine (Michel & Lamas, 1992; Nathan & Xie, 1994; Sessa, 1994). Three isoforms of NOS have been identified to date: neuronal (nNOS), inducible (iNOS) and endothelial (eNOS); the alternative nomenclature NOS-1, -2 and -3, respectively, is also sometimes used. nNOS was originally identified in the brain (Bredt & Snyder, 1990; Mayer, 2000), but has been subsequently found to be also constitutively expressed in a variety of cells, including cells of the spinal cord, adrenal glands, peripheral nitrergic nerves, several types of epithelial cells and kidney macular densa cells (Tracey *et al.*, 1994). iNOS is so named because it was originally identified in macrophages exposed to various cytokines or microbial products (Hevel *et al.*, 1991; Stuehr *et al.*, 1991), and expression is predominantly limited to cells of the immune system and lung epithelial cells following stimulation with cytokines (Asano *et al.*, 1994; Tracey *et al.*, 1994). Low levels of constitutive iNOS expression has been observed in endothelial cells and cardiac and vascular myocytes, although their functional role in these cells remains unclear (Mayer & Andrew, 1998). eNOS was first identified in vascular endothelial cells from bovine aorta (Förstermann *et al.*, 1991; Pollock *et al.*, 1991), but has since been located in kidney tubular epithelial cells, interstitial cells in the colon and in various brain regions (Dinerman *et al.*, 1994; Tracey *et al.*, 1994; Xue *et al.*, 1994).

All three NOS isoforms consist of homodimers, sharing a similar structure with a C-terminal reductase domain, N-terminal oxygenase domain and calmodulin-binding domain (Sessa, 1994). Other discrete binding sites exist for essential co-factors for enzymatic activity: flavin mononucleotide, flavin adenine dinucleotide and tetrahydrobiopterin (Stuehr, 1997). The amount of nitric oxide produced by NOS in tissue is dependent on substrate/co-factor availability and the expression level and enzymatic activity of NOS, with both parameters

regulated differentially between isoforms and between different tissues. Both eNOS and nNOS are constitutively expressed under normal conditions and are highly dependent on increases in intracellular Ca^{2+} (such as following agonist stimulation or membrane depolarisation) and subsequent binding to calmodulin for activity. In contrast, as previously described, expression of iNOS is negligible under normal conditions until induced by cytokines or exposure to various components of microorganisms such as lipopolysaccharide (Förstermann & Mayer, 2000). Furthermore, the binding affinity of iNOS for calmodulin in relatively low intracellular calcium concentrations is sufficiently high that its activity may be considered largely Ca^{2+}-independent and therefore insensitive to changes in intracellular Ca^{2+} (Stuehr, 1997; Stuehr *et al.*, 1991).

The activity of NOS isoforms can be regulated at several levels and varies between isoforms. Perhaps surprisingly, regulation of expression levels appears to be the only mechanism for regulation of iNOS activity. Furthermore, compared with nNOS and eNOS, the V_{max} for nitric oxide production by iNOS is 10 to 100-fold greater (Förstermann & Mayer, 2000). In addition to the acute regulation brought about by changes in intracellular Ca^{2+} concentrations, nNOS activity may be modulated by the extent of subcellular localization into vesicles, associated with protein-protein interactions with membrane-bound proteins such as postsynaptic density protein PSD-95 (Brenman *et al.*, 1996; Schepens *et al.*, 1997). Similarly, while changes in intracellular Ca^{2+} concentrations represent the predominant mechanism for acute modulation of eNOS activity, subcellular targeting of the enzyme to discrete locations (caveolae) on the plasmalemmal and Golgi membranes has been identified as playing a key role in determining its activity (Garcia-Cardena *et al.*, 1996; Shaul *et al.*, 1996). Such post-translational targeting is thought to be primarily mediated by myristoylation and palmitoylation at specific amino acid residues (Busconi & Michel, 1993; Liu *et al.*, 1995). Once located to caveolae, protein-protein interactions with caveolin-1 and -3 may also regulate eNOS activity

(Bucci *et al.*, 2000; Garcia-Cardena *et al.*, 1996).

Cellular mechanism of action of nitric oxide

Stimulation of soluble guanylate cyclase (sGC) as the main mechanism of action of nitric oxide was in fact demonstrated before its identity as endothelium-derived relaxing factor (EDRF) (Katsuki *et al.*, 1977). In the 30 years since this discovery much has been learnt regarding the complexity of the various cellular mechanisms through which nitric oxide may exerts its physiological effects - the predominant cellular mechanisms thought to mediate its vascular effects are summarised in Figure 1-3 (Denninger & Marletta, 1999). Stimulation of sGC results in an increase in cyclic guanosine monophosphate (cGMP), which can act directly on voltage-gated Ca^{2+} channels to decrease Ca^{2+} influx therefore reducing cytosolic Ca^{2+} concentration leading to vascular relaxation (Blatter & Wier, 1994).

Inhibition of Ca^{2+} influx can also be achieved through the direct action of nitric oxide on Ca^{2+}-dependent K^+ channels, subsequent membrane hyperpolarisation and closure of voltage-gated Ca^{2+} channels (Bolotina *et al.*, 1994; Robertson *et al.*, 1993). In vascular smooth muscle, potassium channels are recognised as playing a predominant role in maintenance of resting membrane potential. This is highlighted by the close association between the overall resting membrane potential (-40 to -55mV) and the equilibrium potential of potassium (E_K) in vascular smooth muscles (approximately -84 mV). In addition, arterial smooth muscles have relatively low K^+ channel density together with low levels of activity, which results in a high resting input resistance (5-15 GΩ). This means that the opening or closure of very few K^+ channels can have a profound effect on membrane potential (Standen & Quayle, 1998). To date, four main types of K^+ channels have been to shown to be expressed in arterial smooth muscle (calcium sensitive, K_{Ca}; ATP-sensitive, K_{ATP}; voltage-sensitive, K_V and inward rectifying, K_{IR}) with their relative contribution to the resting membrane potential varying considerably between different tissues

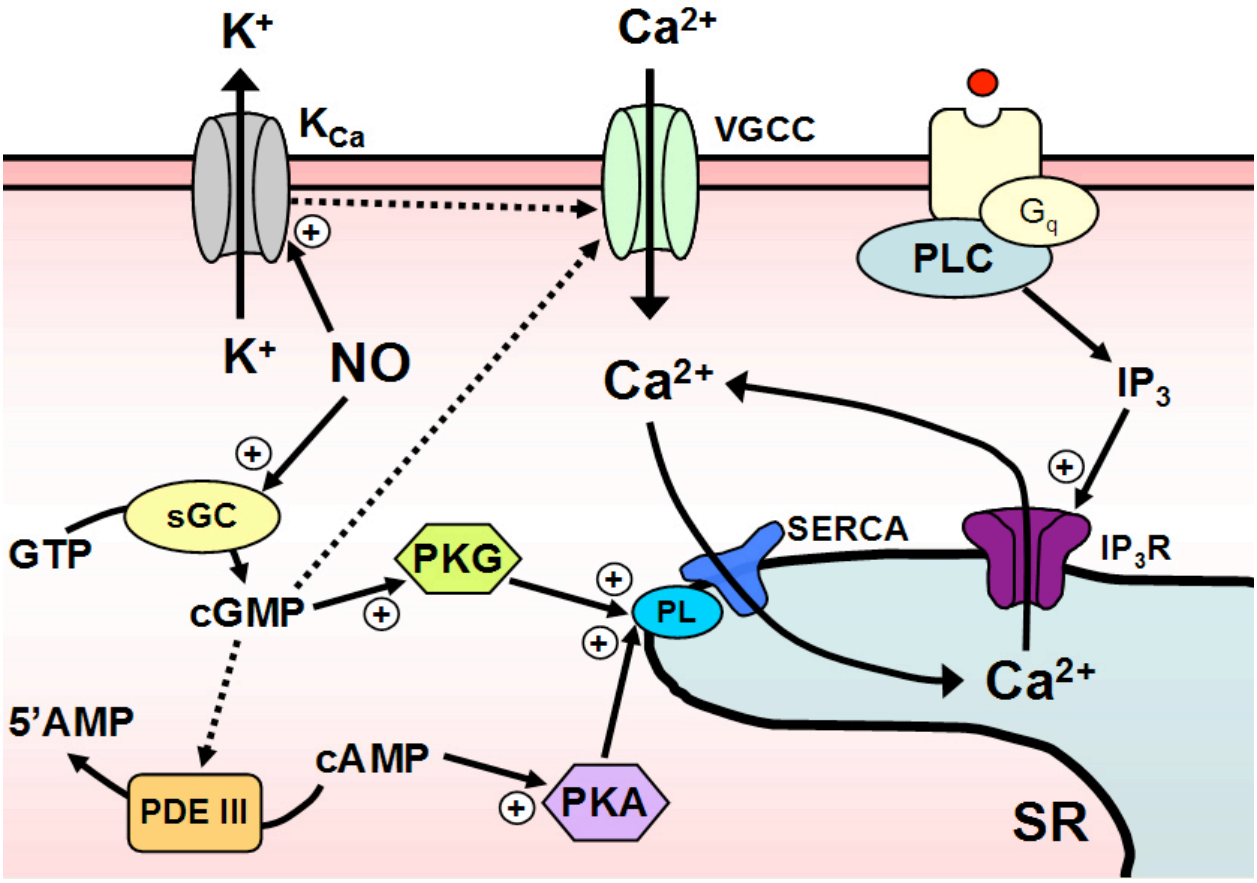

Figure 1-3 - Schematic illustration of the major intracellular mechanisms of action of the vascular effects of nitric oxide

The effects of nitric oxide (NO) in vascular smooth muscle cells are mediated predominantly by stimulation of soluble guanylate cyclase (sGC), although some direct actions have been shown. Dotted arrows indicate inhibitory effects. K_{Ca}, calcium-dependent potassium channels; VGCC, voltage-gated calcium channels; G_q, G-protein mediated receptor associated with phospholipase C (PLC); IP_3, inositol 1,4,5-trisphosphate; IP_3R, inositol 1,4,5-trisphosphate receptor; SERCA, sarcoplasmic-endoplasmic reticulum calcium ATPase; PL, phospholamban; SR, sarcoplasmic reticulum; GTP, guanosine triphosphate; cGMP, cyclic guanosine monophosphate; PKG, protein kinase G; 5'AMP, 5'-adenosine monophosphate; cAMP, cyclic adenosine monophosphate; PKA, protein kinase K; PDE III, phosphodiesterase III (adapted with kind permission from Busse & Fleming, 2000).

(Nelson & Quayle, 1995).

K^+ channels that are activated by intracellular Ca^{2+} (K_{Ca} channels) have been identified in most smooth muscle cells. In addition to their calcium sensitivity, some K_{Ca} channels may

also exhibit a degree of voltage-sensitivity, with their activity increasing with membrane depolarisation (Benham *et al.*, 1986). By causing K^+ efflux in response to increased $[Ca^{2+}]_i$, K_{Ca} channels act to hyperpolarise the cell membrane, thus attenuating the force of contraction. At least three types of K_{Ca} channels have been identified based on both single-channel conductance and pharmacological properties. High-conductance (100-300 pS) channels (also known as BK_{Ca} channels) are traditionally blocked by the scorpion venom peptides *charybdotoxin* (ChTX) and *iberiotoxin* (IbTX), but have also been found to be sensitive to low concentrations (<1 mM) of *tetraethylammonium* (TEA) and the mycotoxin *paxilline* (Brayden & Nelson, 1992; Giangiacomo *et al.*, 1992; Li & Cheung, 1999). ChTX may also selectively block intermediate-conductance (25-100pS) channels (IK_{Ca}), and small-conductance (10-20pS) channels (SK_{Ca}) are selectively blocked by the bee venom toxin *apamin* (Blatz & Magleby, 1986). In contrast to BK_{Ca} channels, IK_{Ca} and SK_{Ca} channels do not exhibit voltage sensitivity. In most vascular smooth muscle, BK_{Ca} channels are the main channels responsible for the Ca^{2+}-sensitive current (Nelson & Quayle, 1995); a possible physiological role proposed as providing negative feedback to limit vasoconstriction.

Voltage-dependent potassium (K_V) channels are activated by depolarisation of the membrane, with a threshold potential for considerable opening ranging from –40 to –30mV (Nelson & Quayle, 1995). Some form of voltage-dependent K^+ current (I_{Kv}) has been identified in all vascular smooth muscle, and K_V channels are recognised as playing an important role in the regulation of resting membrane potential and therefore vascular tone. This stems from many observations where selective blockade of K_V channels with 4-aminopyridine (4-AP) results in depolarisation and contraction of many types of vascular smooth muscle (for example: Hara *et al.*, 1980; Knot & Nelson, 1995). In addition, an I_{Kv} has recently been identified that is relatively insensitive to 4-AP (37% block at 20 mM) but sensitive to TEA (IC_{50} =

2.6mM)(Smirnov *et al.*, 2002). The physiological role of K_V channels is unclear; however they may act to limit membrane depolarisation in response to both pressurisation and vasoconstrictors, thus preventing over-constriction and vasospasm (Nelson & Quayle, 1995).

ATP-sensitive potassium (K_{ATP}) channels couple changes in intracellular metabolism to membrane potential - an increase in intracellular ATP results in channel closure (Quayle *et al.*, 1997). Glibenclamide, a selective blocker of K_{ATP} channels has been shown to cause depolarisation and contraction of several types of vascular smooth muscle, indicating that K_{ATP} channels play a role in the regulation of membrane potential in vascular smooth muscle cells (Beech *et al.*, 1993; Jackson, 1993).

In contrast to the K^+ channels mentioned above, inward rectifier potassium (K_{IR}) channels are responsible for inward K^+ currents. K_{IR} channels are dependent on membrane potential, but in contrast to K_{Ca} and K_V channels, are activated by membrane hyperpolarisation (Quayle *et al.*, 1993). In addition, increased extracellular K^+ concentration causes a rightward shift in the activation potential, and also causes increased conductance. The physiological role of K_{IR} channels in smooth muscle is not entirely unclear, but is thought to include regulation of resting membrane potential in certain vascular tissue, such as cerebral and coronary arteries. In addition, the coupling of blood flow to metabolic demand (i.e. through sensitivity of extracellular K^+) has also been proposed (Nelson & Quayle, 1995).

Increases in intracellular cGMP also results in the activation of cGMP-dependent protein kinase (PKG), which in turn leads to the phosphorylation of proteins in the sarcoplasmic reticulum (SR) involved in sequestration of Ca^{2+} from the cytoplasm to the SR (such as phospholamban). This process is more relevant to situations where smooth muscle contraction is less dependent on Ca^{2+}-influx through voltage-gated Ca^{2+} channels, such as following

agonist-induced activation of the phospholipase C/inositol 1,4,5-triphosphate (IP_3) pathway (Sausbier *et al.*, 2000; Schlossmann *et al.*, 2000).

Increases in intracellular cGMP also modulate the signalling pathways of other cyclic mononucleotides involved in regulation of vascular tone; phosphodiesterase (PDE) III is inhibited by physiologically relevant concentrations of cGMP, therefore reducing the hydrolysis of cyclic adenosine monophosphate (cAMP)(Komas *et al.*, 1991). Elevations in intracellular cAMP concentration lead to activation of protein kinase A, which in turn produce relaxation of vascular smooth muscle (Sandner *et al.*, 1999).

A further mechanism by which nitric oxide can control vascular tone is through modulation of the production of other endothelium-derived vasoactive mediators. Increases in both cyclo-oxygenase and prostaglandin H synthase activity have been observed (via a cGMP-independent mechanism) in response to increased nitric oxide levels, resulting in elevated PGI_2 synthesis and release (Davidge *et al.*, 1995; Salvemini *et al.*, 1993; Salvemini *et al.*, 1994). Nitric oxide, both directly and through the above stimulation of PGI_2 synthesis, also appears to play a key role in the negative feedback control of endothelin-1 release (Boulanger & Luscher, 1990; Saijonmaa *et al.*, 1990).

Vascular effects of nitric oxide

It comes as no surprise that the physiological effect that led to its discovery – relaxation of vascular smooth muscle – is by far the most readily observable effect of nitric oxide on the cardiovascular system (Ignarro *et al.*, 1987; Palmer *et al.*, 1987). The essential contribution of this effect to the regulation of TPR was demonstrated when inhibition of its synthesis resulted in increases in mean arterial blood pressure in excess of 20 mmHg (Rees *et al.*, 1989). In the past two decades, a vast amount of basic and clinical research has demonstrated not only a global

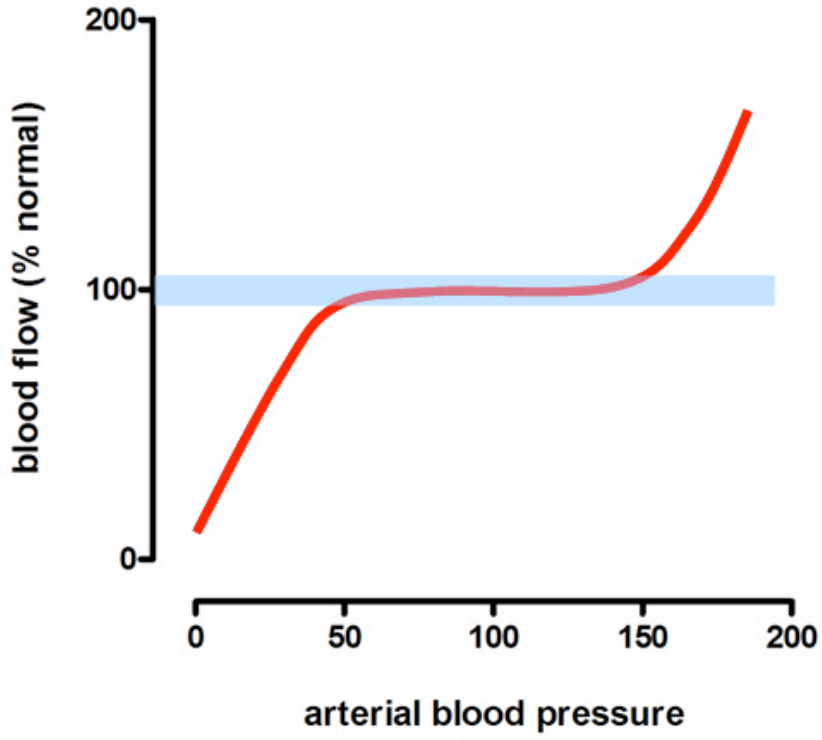

Figure 1-4 - Flow-pressure curve illustrating autoregulation of the cerebral circulation
Autoregulation by the cerebral vasculature maintains a stable blood flow (approximately 700 ml/min in healthy adults) - in spite of changes in mean arterial pressure (MAP) - between the range of ~60 and ~150 mmHg. Outside of these limits of autoregulation, cerebral blood flow rises or falls proportionally with MAP (adapted with kind permission from (Franco Folino, 2007).

role for nitric oxide in the control of systemic vasculature tone and TPR, but in the precise local control of tissue perfusion in different vascular beds (Busse *et al.*, 2000).

With the exception of the lungs, most vascular beds exhibit some degree of autoregulation i.e. the adjustment of blood flow in response to changes in metabolic demand and/or perfusion pressure. Flow is regulated through increases or decreases in vascular resistance, which is turn is determined by the balance of vasoconstrictor and vasodilator tone (Rowell, 1993). For the majority of vascular beds, intrinsic vasoconstrictor tone is generated

primarily through basal myogenic contraction in response to transmural pressure, while vasodilator tone arises from either eNOS-derived nitric oxide or, in the case of terminal arterioles and capillaries, vasodilator metabolites of cellular energy metabolism (Busse *et al.*, 2000). It is clear therefore, that changes in nitric oxide production in response to disease or changes in environment can have significant effects on the local regulation of perfusion of a particular vascular bed.

The cerebral circulation is exquisitely sensitive to changes in MAP, and correspondingly exhibits a high degree of autoregulation in comparison to most other vascular beds (Franco Folino, 2007; Schondorf *et al.*, 2001; Strandgaard & Paulson, 1984). As shown in Figure 1-4, cerebral flow is maintained over the MAP range of approximately 60 to 150 mmHg. At pressures higher than normal (~100 mmHg) myogenic tone increases vascular resistance (autoregulatory vasoconstriction), while at lower pressures decreases in vascular resistance (autoregulatory vasodilatation) maintain cerebral flow (Preckel *et al.*, 1996; Tanaka, 1996; Tanaka *et al.*, 1993).

1.2.3.2 Evidence for adaptation of L-arginine/nitric oxide following simulated microgravity

Following the initial observation by Sangha and co-workers (Sangha *et al.*, 2000) that increased nitric oxide production may account for hyporesponsiveness to vasoconstrictors in isolated arterial rings from 20 day HDT rats, findings from studies attempting to identify the physiological significance and mechanism of changes in vascular nitric oxide signalling have proved to be fairly inconsistent. Protein expression studies investigating levels of the three known nitric oxide synthase (NOS) isoforms following 20 days of HDT revealed an increase in iNOS expression in the aorta relative to with control animals (Vaziri *et al.*, 2000); perhaps surprisingly, no changes in nNOS or eNOS were observed. A more thorough study measuring NOS expression in a range of different arteries demonstrated that both iNOS and eNOS

expression was increased in the carotid arteries and thoracic aorta, decreased in the mesenteric artery and unchanged in the cerebral and femoral arteries (Ma *et al.*, 2003).

Attempts to correlate these findings with functional changes utilising the iNOS inhibitors aminoguanidine or 2-amino-dihydro-6-methyl-4H-1,3-thiazine (AMT) appear to agree with the hypothesis extended by this group (and others) that excess nitric oxide produced by iNOS contributes to the vascular hyporesponsiveness and hypotension following simulated microgravity (Eatman *et al.*, 2003b; Sangha *et al.*, 2000; Vaziri *et al.*, 2000). Unfortunately, both these studies suffer from a poor choice in the inhibitors of iNOS utilised. Compared to other commercially available iNOS inhibitors such as N-(3-(aminomethyl)benzyl)acetamidine (1400W), both aminoguanidine and AMT are significantly less selective for iNOS over eNOS, particularly when used *in vivo* (Garvey *et al.*, 1997; Laszlo *et al.*, 1995). This becomes evident by the fact that in both microgravity studies mentioned above, the iNOS inhibitor used produces a marked pressor response in control animals. As iNOS is not thought to play a significant role in regulation of vascular tone in normal animals (Busse *et al.*, 2000; Kadowitz & McNamara, 2000; Moncada & Higgs, 2006), the pressor response is presumably due to non-selective actions, i.e. inhibition of eNOS. The lack of selectivity (less than 30-fold for iNOS over eNOS) and potency (doses as high as 400 mg/kg have been reported for aminoguanidine) *in vivo* therefore limits the usefulness of aminoguanidine and AMT (and indeed most other 'reasonably' selective iNOS inhibitors including the S-alkylated isothiourea class of compounds) for whole animal cardiovascular research (Cameron & Cotter, 1996; Garvey *et al.*, 1997; Kihara *et al.*, 1991; Southan & Szabo, 1996).

Consistent with the differential structural and functional adaptation in relation to the anatomical location of the artery, there is evidence to suggest that changes in NOS expression may also vary in this way. Wilkerson and colleagues observed enhanced basal tone of middle

cerebral arteries in 2 week HDT rats which was associated with decreased expression of eNOS compared with control animals (Wilkerson *et al.*, 2005). Furthermore, cerebral vascular resistance was also found to be increased during normal posture and during orthostatic challenge. Together with alterations in EDHF-mediated control of cerebral vascular tone, Prisby and colleagues demonstrated significant decreases in nitric oxide-mediated endothelial-dependent vasodilatation in middle cerebral arteries from 14 day HDT rats (Prisby *et al.*, 2006). Given the role that nitric oxide plays in cerebral artery autoregulatory vasodilatation (Jones *et al.*, 2003; Leffler *et al.*, 2000; Tanaka *et al.*, 1993; White *et al.*, 2000), these findings have obvious implications as potential mechanisms for the inadequate cerebral perfusion experienced during orthostatic challenge following exposure to microgravity.

Given the relatively short timeframe since a potential role for nitric oxide in cardiovascular deconditioning was proposed, human studies specifically investigating changes in the L-arginine/nitric oxide pathway following either spaceflight or HDBR are extremely scarce. Using a common technique for the assessment of endothelial function, (post ischaemic hyperaemia following occlusion), flow-dependent vasodilatation in brachial arteries was found to be enhanced following HDBR compared with controls. Furthermore, the increase in flow-dependent vasodilatation was associated with a decrease in post-bed-rest duration of orthostatic tolerance (Bonnin *et al.*, 2001). Interestingly, the vasodilator response to the nitric oxide donor glyceryl trinitrate was unchanged between control and HDBR groups. Although changes in nitric oxide function were not measured directly in this study, as nitric oxide is thought to be the predominant mediator of flow-induced arterial vasodilatation these findings provide evidence that simulated microgravity may cause adaptation of the regulation of peripheral vascular tone by the L-arginine nitric oxide pathway, but not in the vascular effector mechanism of nitric oxide (such as activation of sGC, as discussed above). A lack of difference in response to the

nitric oxide donor sodium nitroprusside (SNP) has also been demonstrated in rats following HDT (Ma *et al.*, 1998a).

1.2.4 Summary of observations from human studies and animal models

It is clear from the findings discussed above that microgravity (actual or simulated) brings about significant changes in cardiovascular function, and that most (if not all) of the components of the cardiovascular system are affected to a certain degree. Furthermore, the main consequence of such cardiovascular deconditioning – orthostatic intolerance – is likely to be heterogeneous in its aetiology. Presyncopal symptoms (including light-headedness and dizziness) during orthostatic tolerance testing following spaceflight or HDBR have been associated with at least three different haemodynamic profiles: presyncope associated with sudden hypotension, suggestive of a vasovagal response; presyncope following a slow, steady drop in arterial blood pressure; and presyncope in the absence of hypotension (Buckey *et al.*, 1996; Pavy-Le Traon *et al.*, 2007; Watenpaugh & Hargens, 1996). In the first two proposed haemodynamic profiles, orthostatic intolerance may be considered a direct result of (and even synonymous with) orthostatic hypotension – defined clinically as a fall in systolic and diastolic blood pressures of greater than 20 mmHg and 10 mmHg, respectively (Medow *et al.*, 2008). However, the third profile relates to inadequate cerebral perfusion despite maintained arterial pressure – this has been proposed as being a unique mechanism of orthostatic intolerance as a result of cardiovascular deconditioning following exposure to microgravity environments (Watenpaugh & Hargens, 1996; Zhang, 2001). These findings may reflect individual variations in the adaptation of different components of cardiovascular function to microgravity exposure.

While orthostatic tolerance testing in animals remains problematic due to the fundamental cardiovascular differences between bipeds and quadrupeds, many of the cardiovascular adaptations observed in human studies may still be reproduced through use of the

HDT animal model. From such studies, valuable insights into potential mechanisms of cardiovascular deconditioning in response to microgravity have been made. Changes in body fluid volume and autonomic cardiovascular control are likely important contributing factors, however, for the purpose of this thesis, observations demonstrating several forms of arterial adaptation form the premise for the research presented herein. Primarily from the studies using animal models described above, the arterial system has been shown to exhibit at least three different types of adaptation in response to simulated microgravity (Purdy *et al.*, 2003):

- altered vasodilator/vasoconstrictor responsiveness of peripheral arteries associated with structural remodelling;
- altered vasodilator/vasoconstrictor responsiveness of peripheral arteries associated with functional changes to the endothelium and/or vascular smooth muscle;
- altered vasodilator/vasoconstrictor responsiveness of cerebral arteries associated with functional changes to the endothelium and/or vascular smooth muscle.

The scope of this thesis is limited to a particular aspect of arterial adaptation to microgravity; the role of the L-arginine/nitric oxide pathway in the functional adaptation leading to altered vasodilator/vasoconstrictor responsiveness in both the systemic and cerebral vasculatures.

1.3 RESEARCH OBJECTIVES & EXPERIMENTAL DESIGN

1.3.1 Overall hypothesis

Differential adaptation of the vascular L-arginine/nitric oxide pathway occurs in arteries in response to simulated microgravity, such that an increased contribution of endothelium-derived nitric oxide to vascular tone is observed in the peripheral vasculature (caudal to the

hydrostatic indifference point), while a decreased contribution of endothelium-derived nitric oxide to vascular tone is observed in the cerebral vasculature (cephalic to the hydrostatic indifference point).

1.3.2 Specific research objectives

In order to test the above overall hypothesis, specific objectives with discrete hypotheses to test were established:

A. To determine if differential adaptation to simulated microgravity between cephalic (cerebral) and peripheral/caudal vascular beds is observed in vivo. In order to investigate differential changes in the control of vascular tone by nitric oxide, it must first be determined if the differential functional changes observed from previous *in vitro* studies - manifested by increases in responsiveness to vasoconstrictors by cephalic arteries and decreases in responsiveness to vasoconstrictors in peripheral arteries (Delp *et al.*, 1993; Sangha *et al.*, 2001; Zhang, 2000; Zhang *et al.*, 2001) - are reflected by the HDT model *in vivo*. It was therefore hypothesized that the vasoconstrictor response to the selective α_1-adrenoceptor agonist methoxamine is increased in the cephalic arterial vasculature, but decreased in the peripheral/caudal arterial vasculature following exposure to simulated microgravity. Any observed difference observed in methoxamine-induced constriction *in vivo* were also investigated *in vitro* to determine if they reflect direct changes to α_1-adrenoceptor function or were due to increased vasodilator tone. Moreover, to determine if the absence of any change in adrenergic vasodilator responsiveness observed previously *in vitro* (Fadiukova *et al.*, 2005; Ma *et al.*, 1998b) was also observed *in vivo*, the effects of the selective β_2-adrenergic agonist salbutamol on the vascular tone of the cephalic and peripheral/caudal arterial vasculature following 14 day HDT were also investigated.

B. To determine if any observed differential adaptation to simulated microgravity between cephalic (cerebral) and peripheral/caudal vascular beds in vivo reflects changes in the contribution of nitric oxide to vascular tone. Based on previous in vitro observations any differences in the responsiveness to vasoconstrictor stimuli observed between cephalic and peripheral/caudal vasculature following HDT may reflect differential changes in the L-arginine/nitric oxide pathway (Jasperse *et al.*, 1999; Schrage *et al.*, 2000; Vaziri *et al.*, 2000; Wilkerson *et al.*, 2005). It was hypothesized that the vasodilator response to agonist-induced stimulation of nitric oxide and/or activation of sGC by acetylcholine and SNP, respectively, would be decreased in the cephalic arterial vasculature, but increased in the peripheral/caudal arterial vasculature. Furthermore, the vasoconstrictor response to inhibition of NOS isoforms by the non-selective NOS inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME) and the highly selective iNOS inhibitor 1400W would be decreased in the cephalic arterial vasculature, but increased in the peripheral/caudal arterial vasculature.

C. To determine the level at which any observed changes in the L-arginine/nitric oxide pathway in response to simulated microgravity occurs. Any observed changes in the nitric oxide-mediated control of vascular tone in the cephalic or peripheral/caudal vasculature may reflect changes to the L-arginine/nitric oxide pathway at several levels, including changes in NOS protein expression and activity, rate of removal of nitric oxide, and the cellular effector mechanism (activation of sGC)(Busse *et al.*, 2000; Moncada & Higgs, 2006; Sangha *et al.*, 2000). It was hypothesised that NOS protein expression and/or activity was decreased in the cephalic arterial vasculature, but increased in the peripheral/caudal arterial vasculature. Furthermore the observed differences in the control peripheral vasculature tone are not through changes in the pharmacokinetics (i.e. rate of removal) or pharmacodynamics (i.e. duration of vasodilator effect) of nitric oxide.

1.3.3 Experimental design

Validation & in vivo characterisation of a simulated microgravity model. The HDT rat model is the most widely used animal model for investigating cardiovascular adaptation to simulated microgravity (Morey *et al.*, 1979). Despite the obvious limitations of using a quadruped species such as rat to simulate a condition experienced primarily by humans, this model is accepted as one of the more suitable for use in a research laboratory environment. Following the establishment of a modified version of the 14 day HDT model in our laboratory, demonstration of functional adaptation to simulated microgravity was required before proceeding with further studies. In this regard, the application of orthostatic stress to the whole animal was considered most appropriate to determine any differences between control and HDT animals. Several attempts to employ a 'head-up tilt' procedure (equivalent to the most common clinical test to evaluate orthostatic intolerance in humans) were plagued with technical difficulties; an alternative method for subjecting the animals to orthostatic stress was therefore employed i.e. the use of a lower body negative pressure (LBNP) chamber. This procedure is also used for the assessment of orthostatic tolerance in humans (Convertino, 2001; Tobal *et al.*, 2001), and therefore was deemed an appropriate method for such investigation in animal models. To demonstrate functional adaptation of the peripheral arterial vasculature following exposure to simulated microgravity, the effects of LBNP on MAP in anaesthetised animals were investigated. Furthermore, to assess whether changes in autoregulation of cerebral blood flow may be observed *in vivo* following HDT, the conductance of the vascular bed distal to the common carotid artery ($C_{carotid}$) was measured as an index of intrinsic cerebrovascular tone in response to changes in MAP produced by LBNP. In addition, to determine if any differences in MAP responses following HDT are associated with hypovolaemia, total blood volume was also measured.

A. Assessment of tone in the cephalic, peripheral and caudal vascular beds in response to adrenergic receptor stimulation. To determine if the vasoconstrictor response to methoxamine was differentially altered between cephalic, peripheral and caudal vascular beds following HDT, the dose-response relationship between methoxamine and $C_{carotid}$, MAP and the conductance of the vascular bed distal to the common iliac artery (C_{iliac}; index of intrinsic hind limb vascular tone) was measured in control and HDT animals. Similarly, to determine if the vasodilator response to salbutamol is differentially altered between cephalic, peripheral and caudal vascular beds following HDT, the dose-response relationships for salbutamol and $C_{carotid}$, MAP and C_{iliac} were measured. To determine if any changes in the *in vivo* response to methoxamine reflected direct changes to α_1-adrenoceptor function or are due to increased vasodilator tone, the concentration-response relationship to methoxamine in isolated carotid, superior mesenteric and iliac artery rings from control and HDT animals was measured. Furthermore, α_1-adrenoceptor reserve was determined as part of the same study to assess whether any observed changes in the concentration-response to methoxamine were due to alterations in α_1-adrenoceptor expression/membrane trafficking.

B. Assessment of tone in the cephalic, peripheral and caudal vascular beds in response to drugs that interact with the L-arginine/nitric oxide pathway. Using the same experimental protocol as *A*, changes in the intrinsic vascular tone of cephalic, peripheral and caudal vascular beds in response to pharmacological manipulation of the L-arginine/nitric oxide pathway were assessed by comparing the dose-response relationships between $C_{carotid}$, MAP and C_{iliac} and acetylcholine, SNP, L-NAME and 1400W between control and HDT rats.

C. Assessment of changes in NOS protein expression, NOS enzyme activity and pharmacokinetics/pharmacodynamics of nitric oxide. The effects of HDT on NOS expression were assessed through semi-quantification of eNOS and iNOS protein in carotid, mesenteric and

iliac arteries isolated from control and HDT rats through the use of western blotting. Any changes in spatial expression were also assessed from the immunohistochemical staining of eNOS and iNOS in carotid, mesenteric and iliac arteries isolated from control and HDT rats. Taking advantage of a new technique for the measurement of nitric oxide I recently established in the laboratory, differences in NOS activity between carotid and iliac arteries from HDT and control animals was assessed by the electrochemical measurement of acetylcholine-stimulated nitric oxide production in the presence and absence of L-NAME and 1400W. The same method for electrochemical detection of nitric oxide was modified for the measurement of nitric oxide concentrations in circulating blood in response to intravenous infusions of SNP. The high degree of temporal resolution afforded by this technique allowed the pharmacokinetic half-life of nitric oxide in the circulation to be measured. Simultaneous measurement of MAP during the same SNP infusion allowed measurement of the pharmacodynamic half-life (from the rate of decline of depressor response to SNP).

2 MATERIALS & METHODS

2.1 EXPOSURE TO SIMULATED MICROGRAVITY

Male Sprague-Dawley rats (230±15 g) were randomly assigned to either singly-housed control or 14 day HDT groups. HDT was achieved by utilizing a modification of the HLU technique developed at the AMES NASA research centre (Morey *et al.*, 1979), involving the application of a custom built tail harness consisting of a 40 mm x 80 mm section of Akton® viscoelastic polymer padding (7 mm thick; Action Products, USA) surrounded by a 80 mm long piece of plastic tubing (19 mm ID; Fisher Scientific, USA). The tail harness was attached via steel wire to a swivel clip mounted on a steel cross bar and the height of the cross bar could be adjusted so that the angle of HDT was maintained at 30° HDT as the rat increased in size over the course of the 14 days. Rats were able to roam the cage freely using their front paws and were provided with standard laboratory rodent diet and water *ad libitum*. All procedures were approved by the University of British Columbia Institutional Animal Care and Use Committee (APPENDIX 1).

2.2 *IN VIVO* ASSESSMENT OF VASCULAR FUNCTION

2.2.1 Surgical preparation

Following either 14 days of HDT or control housing, animals were prepared for acute cardiovascular measurements. Anaesthesia was induced via intraperitoneal injection of sodium thiobutabarbital (100 mg/kg) and additional 10 mg/kg intraperitoneal injections were performed where necessary until a surgical plane of anaesthesia was achieved. Due to the long acting nature of sodium thiobutabarbital, this initial dose was often sufficient to maintain anaesthesia for the entire duration of the experiment. Where necessary, an additional 5 mg/kg dose was

administered via the intravenous cannula to maintain an adequate depth of anaesthesia. For all experimental protocols, the following procedures were performed:

- Tracheotomy followed by cannulation of the trachea, to ensure a consistently patent airway.
- Cannulation of the left jugular vein with polyethylene tubing (PE-50; Beckton Dickinson, USA). A T-piece connector was placed approximately 3 cm from the site of entry into the jugular vein to allow intravenous injection and/or infusion of drugs simultaneously with saline infusion.
- Cannulation of the left common carotid artery with PE50 tubing filled with 25 IU/ml heparinized 0.9 % saline, to allow measurement of arterial pressure via a pressure transducer (P23DB; Gould Statham, USA) connected to a bridge amplifier (ETH-260; C.B. Sciences Inc., USA).

Core body temperature was monitored and maintained at 37±0.4 °C using a rectal probe and heating pad attached to a temperature controller (Model 71; Yellow Spring Instruments, USA). Fluid loss due to evaporation at surgical sites was replaced with an intravenous infusion of 0.9 % saline solution at a rate of 5 ml/kg/hour using a Pump 11 Plus type syringe pump (Harvard Apparatus, USA). Additional surgical procedures were performed as described for individual experimental protocols. Following completion of all surgical procedures, a 1 hr equilibration period was allowed before commencing an experimental protocol.

2.2.2 Protocol I – Haemodynamic response to LBNP

Anaesthetized animals were placed in a custom-built plexiglass chamber modified so that measured degrees of negative pressure could be applied (adapted from the technique described in reference Bedford & Tipton, 1985). An airtight seal was made using a latex sheath

at the level of ~1 cm caudal to the xyphoid process, so that only the lower body of the animal was subjected to negative pressure. When the animal was appropriately positioned, the right common carotid artery was located and cleaned of connective tissue to allow measurement of carotid artery flow ($Q_{carotid}$) using an ultrasonic transit-time volume flow probe (2-PSB type) connected to a T206 small animal flowmeter (Transonic Systems, USA). After equilibration, the haemodynamic response (as measured by changes in MAP and $Q_{carotid}$) to increasing degrees of negative pressure (0 to -8 mmHg relative to ambient pressure) applied to the chamber was measured.

2.2.3 Protocol II - Haemodynamic response to vasoactive drugs

In addition to measurement of $Q_{carotid}$ as described in protocol I, iliac artery flow (Q_{iliac}) was also measured simultaneously using ultrasonic transit-time flowmetry. A longitudinal medial laparotomy along the lower two thirds of the linea alba was performed, and the right common iliac artery was located immediately distal to the bifurcation of the abdominal aorta. The artery was cleaned of fat and connective tissue to allow adequate positioning of the flow probe for ultrasonic measurements. After equilibration, vascular function (as measured by changes in MAP, $Q_{carotid}$ and Q_{iliac}) was assessed by constructing dose-response curves to the following vasoactive drugs. Unless stated otherwise, drugs were administered as dose-randomised controlled bolus intravenous injections (volume < 0.7 ml per injection) at a rate of 0.79 ml/min using a Pump 11 Plus type syringe pump (Harvard Apparatus, USA):

- methoxamine (0.04-2 μmol/kg)
- acetylcholine (1-200 nmol/kg)
- sodium nitroprusside (1-200 nmol/kg)
- salbutamol† (1 nmol–2 μmol/kg)

- N^{ω}-nitro-L-arginine methyl ester (L-NAME; 1-100 μmol/kg; non-randomised, cumulative)
- N-(3-(Aminomethyl)benzyl)acetamidine (1400W; 0.2-20 μmol/kg; non-randomised, cumulative)

† - L-NAME (50 μmol/kg) was administered intravenously after 30 mins of the 1 hr equilibration period to eliminate the nitric oxide-mediated component of the vascular response to salbutamol reported previously (Wang *et al.*, 1993).

2.2.4 Protocol III - Pharmacokinetics/pharmacodynamics of nitric oxide

Amperometric detection of nitric oxide in circulating blood was performed using electrochemical nitric oxide sensors utilizing a gas permeable membrane selective for nitric oxide (AmiNO-700-T; Innovative Instruments, USA), connected to an Apollo 4000 free radical analyzer (WPI Inc., USA), based on the technique described by Mochizuki *et al.* (Mochizuki *et al.*, 2002; Mochizuki *et al.*, 2003). Briefly, oxidation of nitric oxide at a working electrode held at a positive potential of ~900 mV results in generation of a small redox current that is proportional to the concentration of nitric oxide in solution. Sensors were calibrated using a saturated solution of nitric oxide on each experimental day, prior to measurements being taken. In addition, the integrity (and therefore the selectivity) of the nitric oxide-selective membrane was verified by testing for a lack of response to 10 μM $NaNO_2$ (Wadsworth *et al.*, 2006).

Following induction of anaesthesia and cannulation of vessels for drug infusion/measurement of arterial pressure described above, the right common iliac artery was located and prepared in similar fashion to that described in protocol II. The dimensions of the amiNO-700-T NO sensor (tip diameter 700 μm; cable housing diameter ~900 μm) are such that it may be inserted into the abdominal aorta via cannulation of the right common iliac artery. The tip was advanced retrogradely back along the common iliac artery a short distance past the

bifurcation of the abdominal aorta such that it was located directly in the flow of blood, but did not obstruct blood flow to the left common iliac artery.

After equilibration, the depressor response and change in plasma nitric oxide concentration in response to 2.5 min infusions of sodium nitroprusside at 40, 80 and 100 nmol/kg/min were recorded. From preliminary experiments, 2.5 mins was found to be sufficient for both the concentration of plasma nitric oxide and depressor response to reach steady state. Infusion doses were administered in duplicate for each experiment, with the dose order randomised for each trial. The pharmacokinetic half-life of nitric oxide and pharmacodynamic half-life of the depressor response were calculated from the decay constant determined from fitting a single exponential to the experimental trace at the point following cessation of the sodium nitroprusside infusion until the plasma nitric oxide concentration/MAP returned to baseline, according to equation 1:

$$[\mathrm{NO}] = e^{-kt} \qquad \text{(Eq.1)}$$

where **[NO]** is circulating nitric oxide concentration, ***e*** is the base of the natural logarithm, ***k*** is the decay constant and ***t*** is time.

Due to the nature of *in vivo* amperometric measurements, absolute plasma nitric oxide concentrations are not readily measured. Alternatively, the change in plasma nitric oxide concentration in response to a stimulus (such as infusion with a nitric oxide donor) may be measured. Furthermore, in contrast to *in vitro* measurements using such nitric oxide sensors, where temperature at the site of electrochemical measurement may be more tightly controlled, additional consideration was needed for *in vivo* nitric oxide measurements, as the redox current measured in response to nitric oxide oxidation is significantly dependent on temperature. To

control for this, the temperature dependency of the nitric oxide sensor was assessed as part of the daily calibration procedure; this relationship could be described by a second order polynomial equation which was subsequently applied as a correction factor based on the measured core body temperature of the animal (measured simultaneously, as described above) to the raw signal from the nitric oxide sensor.

2.2.5 Protocol IV - Measurement of blood volume

Total blood volume was measured using the ^{51}Cr-labelled red blood cell method (Gray & Sterling, 1950). Briefly, animals were anesthetized as above and cannulae were inserted into right exterior jugular vein and right common carotid artery for the injection of ^{51}Cr-labelled red blood cells and the withdrawal of a reference blood sample, respectively. After a 30 min equilibration, an aliquot of red blood cells labelled with ^{51}Cr (0.25 ml) containing 180,000±20,000 counts per min (cpm) was injected and a reference blood sample (0.2 ml) was obtained for the measurement of radioactivity at 20 min following injection. The radioactivity contained in the blood samples, syringes used for the injection, and test tubes used for holding the radiolabeled samples was counted with a gamma counter at energy settings of 271−375 keV. Total blood volume was calculated from equation 2:

$$\textit{total blood volume} = \frac{(\textit{total injected cpm} \times \textit{0.2 ml})}{\textit{sample cpm}} \qquad \text{(Eq.2)}$$

where: *total injected cpm* = *total aliquot cpm* – (*syringe* + *test tube cpm*).

2.2.6 Drugs & reagents used for *in vivo* measurements

All drugs and chemicals were obtained from Sigma-Aldrich Chemicals (USA), with the

exception of 1400W which was purchased from Tocris Biosciences (UK) and ^{51}Cr which was obtained from Perkin-Elmer (USA).

2.2.7 Data acquisition and statistical analysis for *in vivo* measurements

The analogue signals from the bridge amplifier/flowmeter/free radical analyzer were digitized using a MacLab 4/s data acquisition system (A.D. Instruments, Australia) and were displayed and recorded using a PC running Chart v4.2 software (A.D. Instruments, Australia) and saved for subsequent offline analysis.

Heart rate (HR) and MAP were determined as the cyclic rate and cyclic mean of the arterial pressure signal, respectively. In order to determine intrinsic vascular tone in particular regional beds, conductance values were calculated to normalize for changes in MAP. This index of vascular tone is preferred over its reciprocal, resistance, in situations where changes in tone lead primarily to changes in flow (Lautt, 1989; Lautt, 1999; O'Leary, 1991). Conductance of vascular beds served by the carotid ($C_{carotid}$) and iliac (C_{iliac}) arteries was calculated as $Q_{carotid}$ or Q_{iliac} divided by MAP. Responses to drugs were measured as the peak change from baseline values for each experimental group, and described as the mean±standard error.

Differences in baseline cardiovascular parameters and total blood volume between groups were analyzed individually for each protocol using students *t*-test with Welch's correction for unequal variances. The relationship between increasing LBNP, MAP, $Q_{carotid}$ and $C_{carotid}$ were compared between control and HDT groups using repeated-measures two-way analysis of variance (ANOVA). Where appropriate, sigmoidal dose-response curves were fitted using Prism v4.03 software (GraphPad, USA) according to equation 3 and individual curves were used to determine the mean efficacy (E_{max}) and potency (ED_{50}) of each drug for the cardiovascular variables of interest.

$$response\ (y) = \frac{bottom + (top - bottom)}{1 + 10^{(\log EC50 - x)HillSlope}} \quad \text{(Eq.3)}$$

Dose-response curves were analyzed statistically using two-way repeated-measures ANOVA, followed by planned Bonferroni post-tests to determine significant differences in E_{max} or ED_{50} between groups. Both the peak change in plasma nitric oxide concentration/peak depressor response to infusion of sodium nitroprusside and the pharmacokinetic half-life of nitric oxide /pharmacodynamic half-life of the depressor response were compared between control and HDT animals using two-way repeated-measures ANOVA.

All statistical analyses were performed using Prism v4.03 with the probability of type I error (α) set at 0.05.

2.3 *IN VITRO* ASSESSMENT OF VASCULAR FUNCTION

2.3.1 Protocol V - Measurement of alpha-adrenoceptor function

Tissue collection & preparation

After 14 days of either HDT or control treatment, animals were anaesthetized with sodium thiobutabarbital (100 mg/kg i.p.). After the onset of surgical anaesthesia, the common carotid, superior mesenteric, and iliac arteries were removed and immediately transferred to a Petri dish containing Krebs solution (composition in mM: NaCl 112.22; KCl 4.5; glucose 11.1; $NaHCO_3$ 26.19; KH_2PO_4 1.2; EDTA 0.0026; $MgCl_2 \cdot 6H_2O$ 1.2; and $CaCl_2 \cdot 2H_2O$ 2.56) gassed continuously with 95% O_2/5% CO_2 at room temperature. The isolated arteries were cleaned of extraneous fatty and connective tissue and sectioned into ~5 mm rings. The arterial rings were mounted in tissue baths containing 20 ml of Krebs solution gassed continuously with 95% O_2-5% CO_2 at 37°C and connected to Grass FT-03-C force-displacement transducers (Grass

Instruments, USA) for measurement of isometric tension development (Bevan & Osher, 1972). The arteries were placed under a preload of 1 g resting tension and allowed to equilibrate for 1 hr before beginning experimental measurements.

In order to characterize the functional α_1-adrenoceptor reserve characteristics in the arteries, cumulative concentration-response curves (CRCs) to methoxamine were constructed before and after a partial irreversible inactivation of receptors by phenoxybenzamine (3 nM, 5 min incubation). Preliminary studies showed that there were no time- or vehicle (0.1 % EtOH)-dependent differences between the methoxamine CRCs before and after vehicle treatment. After equilibration, 80 mM KCl was added to the bath to verify suitable contractile viability of the arteries. After the contractile response to KCl reached a plateau, the arteries were washed three times (10 min between successive washes) and were allowed to relax to a stable baseline. A control CRC to methoxamine was then constructed over the concentration range 1 nM to 1 mM. The concentration of methoxamine was increased in log increments in a cumulative manner. For each concentration, the arteries were allowed to contract to a plateau before the next administration was given. After the first CRC, the arteries were washed four times (10 min between successive washes). Once a stable baseline tension was recovered, the arteries were incubated with 3 nM phenoxybenzamine for 5 min, followed by four washes (15 min between successive washes). Then, a second methoxamine CRC was constructed in a manner identical to the first CRC.

Determination of receptor reserve

The functional dissociation constant (K_A) of methoxamine and the fraction of receptors remaining functional (q) after the phenoxybenzamine treatment were determined by using the graphical analysis of receptor reserve developed by Furchgott (Furchgott, 1966). CRCs to methoxamine were produced in the absence, and following 5 min incubation with 3 mM

phenoxybenzamine. The K_A of methoxamine and the fraction or remaining functional receptors q after phenoxybenzamine treatment were used to construct reciprocal plots of equieffective concentrations according to equation 4:

$$\frac{1}{[A]} = \frac{(1-q)}{q \bullet K_A} + \frac{1}{q \bullet [A']} \qquad \text{(Eq.4)}$$

where $[A]$ and $[A']$ are equieffective concentrations of methoxamine before and after incubation with methoxamine, respectively. The above plot yields a straight line, from which the values of K_A and q can be determined according to equations 5 and 6:

$$K_A = \frac{(slope - 1)}{\text{int}\, ercept} \qquad \text{(Eq.5)}$$

$$q = \frac{1}{slope} \qquad \text{(Eq.6)}$$

Using the obtained values for K_A and q, a receptor occupancy-response plot was constructed (Figure 3-12), calculating the fraction of receptors occupied (RA/Rt) from equation 7:

$$\frac{RA}{Rt} = \frac{[A]}{[A] + K_A} \qquad \text{(Eq.7)}$$

Contractile responses, expressed as % maximal response, were plotted against percentage of receptors occupied (% RA/Rt). The resulting receptor-occupancy-response plot was used to compare the relative receptor reserve contents of a given artery from the HDT and control groups. For quantitative and statistical comparisons of receptor reserve, the fraction of receptors occupied at 50% response levels (effective occupancy 50; EO_{50}) was calculated and its reciprocal was used to define the degree of receptor reserve using equation 8 (Sheys & Green, 1972) :

$$RR = \frac{[EO_{50}] + K_A}{[EO_{50}]} \qquad \text{(Eq.8)}$$

Implicated in the above definition of receptor reserve is that a receptor reserve exists for all values of greater than 2. Therefore, this method allows quantitative representation and comparison of receptor reserves (Purdy & Stupecky, 1984).

2.3.2 Protocol VI – Measurement of eNOS activity of isolated carotid and iliac arteries

Taking advantage of a new technique for the measurement of nitric oxide recently established in our laboratory, electrochemical measurement of nitric oxide production from isolated arteries was performed to determine any differences in NOS activity between common carotid and common iliac arteries from HDT and control animals. This method was used in preference to existing colorimetric (such as the Griess reaction) or radiolabeled L-citrulline assays due to its significantly enhanced sensitivity and lower detection limits of nitric oxide concentrations.

Tissue collection & preparation

Animals were anaesthetised as described above and the right common carotid and right common iliac arteries were isolated, excised, and immediately transferred to a Petri dish containing Krebs solution (composition in mM: NaCl 112.22; KCl 4.5; glucose 11.1; $NaHCO_3$ 26.19; KH_2PO_4 1.2; EDTA 0.0026; $MgCl_2 \cdot 6H_2O$ 1.2; and $CaCl_2 \cdot 2H_2O$ 2.56) aerated continuously with 95% O_2/5% CO_2 at room temperature. The isolated arteries were cleaned of extraneous fatty and connective tissue and cut to a standard length (3 mm for carotid and 2 mm for iliac arteries) in order to minimize variability in surface area of endothelium between experiments. Length-normalised artery sections were mounted on PE-50 polyethylene tubing attached to 23G-1 hypodermic needles (Beckton Dickinson; USA) and perfused with Krebs solution, aerated continuously with 95% O_2/5% CO_2 at 37°C in a custom built chamber (volume ≈ 250 μl) at a flow rate of 1 ml/min using a MPII model peristaltic roller pump (Harvard Apparatus, USA). The concentration of nitric oxide in the perfusate was measured amperometrically by insertion of an AmiNO-700 NO sensor (Innovative Instruments, USA) into the flow of perfusate via a side channel in the custom built chamber, and connected to an Apollo 4000 free radical analyzer (WPI Inc., USA). Sensors were calibrated and tested for specificity as described above. A diagram of the apparatus is displayed in Appendix 2.

Measurement of acetylcholine-induced nitric oxide production

Acetylcholine was infused into the system prior to the peristaltic pump using a Pump 11 Plus type syringe pump (Harvard Apparatus, USA) at infusion rates that resulted in concentrations in the perfusate of 1 nM – 10 μM. Concentration-response curves were generated to acetylcholine in the absence or any inhibitors, and then following a 15 min infusion with either 1400W (2.5 μM) or L-NAME (20 μM).

2.3.3 Drugs & reagents used for *in vitro* measurements

All drugs and chemicals were obtained from Sigma-Aldrich Chemicals (USA), with the exception of 1400W which was purchased from Tocris Biosciences (UK). O_2/CO_2 gas mixture (95%/5%) was obtained from Praxair (Canada).

2.3.4 Data acquisition and statistical analysis for *in vitro* measurements

For isometric tension of isolated artery measurements, the analogue signal from the bridge amplifier was digitized using a BIOPAC Data Acquisition System (MP150CE; BIOPAC Systems, USA) and displayed and recorded using a PC running AcqKnowledge v3.8.1 Software (BIOPAC Systems, USA), and saved for subsequent offline analysis. For amperometric measurement of NO concentration in the perfusate of isolated arteries, the analogue signal from the Apollo 4000 free radical analyzer was digitized using a MacLab 4/s data acquisition system (A.D. Instruments, Australia) and displayed and recorded using a PC running Chart v4.2 software (A.D. Instruments, Australia) and saved for subsequent offline analysis.

Sigmoidal concentration-response curves were fitted using Prism v. 4.03 software (GraphPad, U.S.A.), as described for *in vivo* measurements. Concentration-response curves were compared between control and HDT groups using two-way ANOVA followed by planned Bonferroni post-tests for differences in EC_{50} or E_{max} values, or by repeated measures two-way ANOVA where fitting of sigmoidal concentration-response curves was not justified by the data. Hyperbolic occupancy-response curves were fitted using Prism v. 4.03 software and compared between groups using two-way ANOVA followed by planned Bonferroni post-tests for differences in the receptor occupancy required for 50% of maximal response. Receptor reserves were compared by unpaired, one-way Student's *t*-test with by Bonferroni's correction for multiple comparisons.

All statistical analyses were performed using Prism v 4.03 with the probability of type I error (α) set at 0.05.

2.4 BIOCHEMICAL ASSESSMENT OF NITRIC OXIDE SYNTHASE EXPRESSION

2.4.1 Protocol VII - Semi-quantification of arterial eNOS & iNOS protein expression

Tissue collection & preparation

Expression of eNOS and iNOS proteins were determined in the common carotid, iliac and superior mesenteric arteries. After 14 days of either control housing or HDT, rats were anaesthetized with thiobutabarbital as described above to allow excision of the required arteries. The vessels were isolated, excised, immediately frozen in liquid nitrogen and stored at -80°C for processing at a later date. Artery samples were homogenized with a Kinematica Polytron homogenizer (Brinkmann Instruments, USA) in RIPA lysis buffer (PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 0.004% sodium azide, 1% DMSO, 1% protease cocktail inhibitor, 1% sodium orthovanadate). The homogenates were centrifuged twice at 10,000 g for 10 minutes at 4°C to remove any tissue debris without precipitating suspended proteins. Total protein concentrations in the samples were determined by Bradford dye colorimetric assay against a bovine serum albumin protein standard curve (0.25μg/μL – 1.8 μg/μL).

Western Blotting of eNOS & iNOS

Protein samples containing Laemmli sample buffer and 2-mercaptoethanol were heated for 10 minutes in a water bath (70°C to 80°C). Samples (40 μg total protein) were loaded onto 7.5% Tris-HCl polyacrylamide pre-cast gels along with protein markers, and iNOS and eNOS electrophoresis standards. The protein was size fractioned in Tris/glycine/SDS buffer at 130 V for 90 minutes. Following electrophoresis, proteins were transferred onto a methanol pre-treated

PVDF membrane (Millipore Corporation, USA) at 120 V for 1 hr in cold (4°C) transfer buffer (Tris/glycine/15% methanol). The membrane was blocked overnight at 4°C in blocking buffer (5% nonfat milk in wash buffer containing 50 mM Tris-HCl phosphate-buffered saline (PBS), 0.05% TritonX-100, 0.05% Tween-200). The membrane was then incubated at room temperature for 1 hr in mouse anti-eNOS (1:2000) or anti-iNOS (1:2000) and anti ß-actin (1:2000) monoclonal antibodies and washed for 30 minutes in wash buffer on a shaker, replacing the wash buffer every 10 minutes. This was followed by incubation at room temperature for 1 hr in a fluorescent goat anti-mouse IgG (1:4000). The washing scheme was repeated with an additional final 5-minute wash in distilled water. The membranes were then scanned using an Odyssey Infrared Imaging System (LI-COR Biosciences, USA) to detect for fluorescence emitted by the goat anti-mouse secondary antibodies.

Optical density areas of eNOS, iNOS and actin bands were measured using ImageJ – Image Processing and Analysis in Java program (National Institutes of Health, USA). Densitometric analysis of protein bands was conducted by measuring the optical density (O.D.) against a standard optical density curve (0 – 2.6 O.D.). Optical density profile plots (showing O.D. of all bands in each lane) were constructed after which the area under the curve of eNOS, iNOS and ß-actin bands were integrated. This method is widely employed in western blot analysis (Girish & Vijayalakshmi, 2004; Hu *et al.*, 2003). Relative optical densities were computed by calculating eNOS:β-actin and iNOS:β-actin ratios.

2.4.2 Protocol VIII – Immunostaining of arterial eNOS & iNOS

In addition to semi-quantification of eNOS/iNOS protein expression levels using western blotting, immunohistochemical staining of the two NOS isoenzymes was performed on carotid, mesenteric and iliac arteries obtained from control and 14 day HDT rats. In contrast to western blotting, immunohistochemistry allows only qualitative evaluation of differences in

endothelial protein levels, but with the advantage of having some degree of spatial resolution. Slices were prepared from arteries that had been perfusion-fixed *in situ* with paraformaldehyde and made in paraffin wax. Sections (8 μm thick) of the tissues were cut at -20° C, collected on slides, and fixed with liquid nitrogen-cooled acetone. Immunohistochemical staining for either eNOS or iNOS involved incubation of the sections for 3 min in hydrogen peroxidase (0.88 mol/L) to quench endogenous peroxidase activity; treatment with a blocking solution for 30 min; incubation with monoclonal mouse anti-iNOS or anti-eNOS antibodies (BD Laboratories, USA) for 60 min; incubation with biotinylated panspecific universal secondary antibody for 10 min; and incubated with streptavidin-peroxidase complex for 20 min. A 5 min wash with PBS (0.1 M, pH 7.2) was performed between each step and a solution of 3, 3'-diaminobenzidine (0.5 mg/ml in 0.1 M PBS, pH 7.2; Sigma-Aldrich, USA) was used as the chromogen. Some sections were treated with mouse nonspecific immunoglobulin G instead of the primary antibody to serve as negative controls.

2.4.3 Reagents used for biochemical measurements

All reagents used for western blot experiments were obtained from Bio-Rad Laboratories (USA), with the exception of RIPA lysis buffer (Santa Cruz Biotechnology, USA), iNOS and eNOS electrophoresis standards (Cayman Chemicals, MI, USA), anti-eNOS and anti-iNOS monoclonal antibodies (BD Laboratories, Canada), anti- ß-actin (Sigma-Aldrich, USA) and fluorescent secondary antibody (Invitrogen Detection Technologies, USA).

All reagents for the immunohistochemical staining of arteries were obtained as a kit (Vectastain universal quick kit) from Vector Laboratories (USA) with the exception of anti-eNOS and anti-iNOS monoclonal antibodies (BD Laboratories, Canada), goat-anti mouse IgG secondary antibody with horse-radish peroxidase conjugate and paraformaldehyde (Sigma-Aldrich, USA).

2.4.4 Statistical analysis for biochemical measurements

Data are described as mean±SE. Differences in eNOS:β-actin or iNOS:β-actin ratios for each artery between control and HDT groups were analyzed using two-way ANOVA, followed by planned Bonferroni post-tests if ANOVA revealed any differences. All statistical analyses were performed using Prism v4.03 with the probability of type I error (α) set at 0.05.

3 RESULTS

3.1 *IN VIVO* ASSESSMENT OF VASCULAR FUNCTION

3.1.1 Baseline cardiovascular variables and total blood volume

No statistically significant differences in baseline cardiovascular measurements (HR, MAP, $Q_{carotid}$, Q_{iliac}, $C_{carotid}$, C_{iliac}) were observed between the control and 14 day HDT groups (Table 3-1). Total blood volume also remained unchanged between control and HDT groups (17.6±0.3 vs. 17.1±0.3 ml, respectively).

3.1.2 Response to Lower Body Negative Pressure (LBNP)

To investigate whether the functional response of the systemic and cerebral vasculature to LBNP exposure was altered following 14 days of HDT, MAP, $Q_{carotid}$ and $C_{carotid}$ were measured during exposure to varying degrees of LBNP. A marked decrease in MAP was observed in response to increasing LBNP (Figure 3-1*A*), up to a maximum decrease of -48±6 and -56±6 mmHg in control HDT animals, respectively. The relationship between LBNP and decreasing MAP was not determined to be significantly different between control and HDT animals. LBNP also produced a significant decrease in both $Q_{carotid}$ (Figure 3-1*B*) and $C_{carotid}$ (Figure 3-1*C*; maximum decrease in $Q_{carotid}$ of -6.5±0.7 and -9.0±0.3; maximum decrease in $C_{carotid}$ of -3.7±0.8 and -5.9±0.5 for control and HDT animals, respectively). The relationship between LBNP and both $Q_{carotid}$ and $C_{carotid}$ was determined to be statistically significant (P=0.019 and 0.029, respectively). These results indicate that while the systemic vascular response to LBNP is unaffected by 14 days of HDT, compensatory autoregulatory vasodilatation to preserve flow to the vasculature distal to the common carotid artery is impaired.

3.1.3 Haemodynamic response to vasoactive drugs

3.1.3.1 Methoxamine

To assess any changes in α_1-adrenoceptor function in the systemic, cephalic and caudal vascular beds following exposure to simulated microgravity, the dose-response relationships between the selective α_1-adrenoceptor agonist methoxamine (Bianchi *et al.*, 1959) and MAP, $C_{carotid}$ and C_{iliac} were compared between control and 14 day HDT animals. Methoxamine (0.04-2 μmol/kg) decreased $Q_{carotid}$ and Q_{iliac}, (Table 3-2) and increased MAP (Figure 3-2*C*) for both groups of rats. Methoxamine was found to cause a dose-dependent decrease in $C_{carotid}$ and C_{iliac} for both groups (Figure 3-2*A*&*B*), with E_{max} being increased for $C_{carotid}$ (-3.7±0.6 *vs.* -1.9±0.2 ml/min/100 mmHg; P=0.028), but decreased for C_{iliac} (-2.3±0.2 *vs.* -4.1±0.3 ml/min/100 mmHg; P=0.002) as well as MAP (57±3 *vs.* 77±3 mmHg; P=0.001) in the HDT relative to the control group. No significant differences in ED_{50} for methoxamine were observed between the groups for any of the measured haemodynamic variables. These results suggest that α_1-adrenoceptor mediated vasoconstriction is increased in cephalic vascular beds but decreased in the systemic and caudal beds of the HDT group relative to the control group.

3.1.3.2 Salbutamol

To investigate any functional changes in β_2-adrenoceptor-mediated vasodilatation in the systemic, cephalic and caudal vascular beds following exposure to simulated microgravity, the dose-response relationships between the partially selective β_2-adrenoceptor agonist salbutamol (Kelman *et al.*, 1969) and MAP, $C_{carotid}$ and C_{iliac} were compared between control and 14 day HDT animals. In order to selectively investigate the direct vasodilator function of β_2-adrenoceptors in the vascular beds of interest, the previously reported nitric oxide-mediated component of β_2-adrenoceptor-mediated vasodilatation (Wang *et al.*, 1993) was inhibited by

prior intravenous administration of 50 μmol/kg L-NAME. Salbutamol (1 nmol–2 μmol/kg) decreased $Q_{carotid}$ and Q_{iliac} (Table 3-2) and MAP (Figure 3-3*C*) similarly for both groups of rats. Flow values were normalized by MAP to reveal intrinsic vascular tone in regional beds independent of perfusion pressure. Salbutamol produced a similar dose-dependent increase in $C_{carotid}$ and C_{iliac} (Figure 3-3*A&B*) in both groups with no significant difference in determined E_{max} values (3.2±0.1 *vs.* 3.4±0.2 ml/min/100 mmHg for $C_{carotid}$ and 4.1±0.2 *vs.* 4.0±0.1 ml/min/100 mmHg for C_{iliac}) between control and HDT animals. No significant difference in the ED_{50} of salbutamol between groups was observed for any of the measured haemodynamic variables. These results suggest that direct β_2-adrenoceptor-mediated vasodilatation is unaltered in the systemic, cephalic and caudal vascular beds following 14 days of HDT.

3.1.3.3 Acetylcholine

To assess any changes in endothelium-dependent vasodilator function in the systemic, cephalic and caudal vascular beds following exposure to simulated microgravity, the dose-response relationships between the endogenous cholinergic neurotransmitter acetylcholine (known to stimulate endothelium-dependent vasodilatation via endothelial muscarinic receptors) (Boulanger *et al.*, 1994; van Zwieten & Doods, 1995) and MAP, $C_{carotid}$ and C_{iliac} were compared between control and 14 day HDT animals. Acetylcholine (1-200 nmol/kg) decreased $Q_{carotid}$ and Q_{iliac} (Table 3-2) and MAP (Figure 3-4*C*) for both groups of rats. Flow values were normalized by MAP to reveal intrinsic vascular tone in regional beds independent of perfusion pressure. Acetylcholine produced a dose-dependent increase in $C_{carotid}$ and C_{iliac} (Fig. 2), with E_{max} being decreased in the HDT group compared with control for $C_{carotid}$ (5.5±0.6 *vs.* 9.3±0.6 ml/min/100 mmHg; P=0.001), but increased in the HDT group for C_{iliac} (9.8±0.6 *vs.* 5.6±0.5 ml/min/100 mmHg; P<0.001) and MAP (-66±2 *vs.* -54±1 mmHg; P=0.002). No significant difference in the ED_{50} of acetylcholine between groups was observed for any of the measured

haemodynamic variables. These results suggest that endothelium-dependent vasodilatation in response to acetylcholine in the HDT group was less in the cephalic vascular bed but greater in the systemic and caudal beds, relative to the control group.

3.1.3.4 Sodium nitroprusside

To investigate any functional changes in the vasodilator response to nitric oxide in the systemic, cephalic and caudal vascular beds following exposure to simulated microgravity, the dose-response relationships between the nitric oxide donor sodium nitroprusside (Palmer & Lasseter, 1975) and MAP, $C_{carotid}$ and C_{iliac} were compared between control and 14 day HDT animals. Sodium nitroprusside (1-200 nmol/kg) decreased $Q_{carotid}$ and Q_{iliac} (Table 3-2) and MAP (Figure 3-5*C*) similarly for both groups of rats. Flow values were normalized by MAP to reveal intrinsic vascular tone in regional beds independent of perfusion pressure. Sodium nitroprusside produced a similar dose-dependent increase in $C_{carotid}$ and C_{iliac} (Figure 3-5*A&B*) in both groups with no significant difference in determined E_{max} or values (8.4±0.4 *vs.* 8.1±0.4 ml/min/100 mmHg for $C_{carotid}$ and 7.4±0.4 *vs.* 7.2±0.6 ml/min/100 mmHg for C_{iliac}) between control and HDT animals. No significant difference in the ED_{50} of sodium nitroprusside between groups was observed for any of the measured haemodynamic variables. These results suggest that NO-mediated vasodilatation is unaltered in the systemic, cephalic and caudal vascular beds following 14 days of HDT.

3.1.3.5 L-NAME

To assess any functional changes in NOS-mediated vasodilatation in the systemic, cephalic and caudal vascular beds following exposure to simulated microgravity, the dose-response relationships between the non-selective NOS inhibitor L-NAME (Rees *et al.*, 1990) and MAP, $C_{carotid}$ and C_{iliac} were compared between control and 14 day HDT animals. L-NAME

(1-100 μmol/kg) produced a decrease in $Q_{carotid}$, Q_{iliac} (Table 3-2), $C_{carotid}$ and C_{iliac}, and a dose-dependent increase in MAP for both HDT and control groups (Figure 3-6), with E_{max} being significantly decreased in the HDT group compared with control for $C_{carotid}$ (-4.5±0.2 *vs.* -6.4±0.4 ml/min/100 mmHg; P=0.001), but increased in the HDT group for C_{iliac} (-4.9±0.3 *vs.* -3.4±0.2 ml/min/100 mmHg; P=0.002) and MAP (44±2 *vs.* 31±2 mmHg; P=0.001). No significant difference in the ED_{50} of L-NAME between groups was observed for any variables. These results show that regional influence of NOS was less in the cephalic bed, but greater in the caudal bed, and in the systemic vasculature of HDT rats relative to control animals.

3.1.3.6 N-(3-(Aminomethyl)benzyl)acetamidine (1400W)

To determine whether iNOS contributed to any differences observed in response to non-selective inhibition of NOS with L-NAME in the systemic, cephalic and caudal vascular beds following exposure to simulated microgravity, the dose-response relationships between the selective iNOS inhibitor 1400W (Garvey *et al.*, 1997) and MAP, $C_{carotid}$ and C_{iliac} were compared between control and 14 day HDT animals. As shown in Figure 3-7, 1400W (0.2-20 μmol/kg) was found to have negligible effects on $Q_{carotid}$, Q_{iliac}, $C_{carotid}$, C_{iliac} and MAP in both control and HDT animals, therefore formal statistical comparison of these variables between groups was not justified (or feasible).

3.1.4 Pharmacokinetics/pharmacodynamics of nitric oxide

In addition to investigating vascular adaptation of the L-arginine/nitric oxide pathway at the level of nitric oxide production, the kinetics of both removal of nitric oxide from the circulation (apparent pharmacokinetic half-life) and the termination of the depressor response to nitric oxide (apparent pharmacodynamic half-life) following an infusion of SNP were compared between control and 14 day HDT animals. Infusions of SNP (40-100 nmol/kg/min) produced

dose-dependent increases in plasma nitric oxide concentration and decreases in MAP (Figure 3-8), which were not found to be significantly different between control and HDT animals. Pharmacokinetic and pharmacodynamic half-lives were calculated from the corresponding elimination rate constants determined from the experimental trace following cessation of sodium nitroprusside infusion (example trace displayed in Figure 3-9). Neither pharmacokinetic or pharmacodynamic mean half-life values varied significantly between doses or between groups (Figure 3-10). The mean half life of nitric oxide in plasma was determined as 44±3 sec and 41±3 sec and the mean half life of the depressor response was 19±2 sec and 19±1 sec, for control and HDT animals, respectively.

From these data, it appears that 14 days of HDT had no significant effect on the pharmacokinetics or pharmacodynamics of exogenously applied nitric oxide in the systemic circulation. This allows a reasonable assumption that the bioavailability of endogenously produced nitric oxide, and the mechanisms involved the termination of action of the effector mechanism (i.e. sGC stimulation) is similarly unaffected by simulated microgravity.

3.2 *IN VITRO ASSESSMENT OF VASCULAR FUNCTION*

3.2.1 α_1-Adrenoceptor function in isolated carotid, mesenteric and iliac arteries

To further investigate the *in vivo* findings regarding altered haemodynamic responses to the α_1-adrenoceptor agonist methoxamine, isometric contractile responses to methoxamine and α_1-adrenoceptor receptor reserve were determined in control and 14 day HDT rats. In isolated arteries, methoxamine produced a concentration-dependent increase in tension (Figure 3-11). The values for E_{max} were significantly lower in HDT animals compared with control in the carotid (1.1±0.1 vs. 0.8±0.1 g; P=0.033) and mesenteric arteries (1.2±0.1 vs. 0.9±0.1 g;

P=0.012), but were unchanged in the iliac artery (1.3±0.1 vs. 1.2±0.1 g; *P*=0.193). No significant differences in EC_{50} values were observed between control and HDT animals in any of the arteries investigated.

Incubation with phenoxybenzamine (3 nM) for 5 min shifted the methoxamine concentration-response curves to the right. As described in the *Materials & Methods* section, the magnitude of this shift was subsequently used to determine the fraction of remaining functional α_1-adrenoceptors (*q*) and the functional dissociation constant (pK_A), values for which are summarised in Table 3-3. These values were used to construct a receptor occupancy-response plot for each artery (Figure 3-12); α_1-adrenoceptor reserve was determined as the reciprocal of receptor occupancy at 50% of the maximum response (Table 3-4). From the hyperbolic characteristics of the receptor occupancy-response curves determined for the carotid and iliac arteries from both groups of animals (Figure 3-12*A*&*C*), substantial α_1-adrenoceptor reserve was qualitatively apparent. No significant difference between the α_1-adrenoceptor reserve values for iliac arteries from HDT and control groups (14.6±2.9 vs. 20.3±4.5) was observed, however, there was a statistically significant decrease in α_1-adrenoceptor reserve in carotid arteries taken from HDT animals compared with control (6.4±1.7 vs. 15.2±3.2; *P*=0.020). The linear nature of the receptor occupancy-response curves from mesenteric arteries (Figure 3-12*B*) suggested negligible or no apparent receptor reserve in either group - this was confirmed by similar determined receptor reserve values of 2.4±0.4 and 2.8±0.3 in control and HDT groups, respectively.

This study suggests that the maximal contractile response to methoxamine is decreased in carotid and mesenteric arteries following simulated microgravity, and that decreased α-adrenoceptor reserve may account for such changes in the carotid, but not mesenteric arteries. No significant change in maximal contractile response or α-adrenoceptor reserve are observed in

iliac arteries. These findings are in contrast to the *in vivo* observations from study 2, where measurement of carotid and iliac vascular conductance (as an index of intrinsic vascular tone) suggested that there was increased and decreased α-adrenoceptor-mediated vasoconstriction in the carotid and iliac vascular beds, respectively. A possible explanation for this inconsistency between results is that changes in vascular function induced by simulated microgravity may vary depending on the size of blood vessel, as conduit arteries (such as the carotid) are recognised as having significant functional differences (i.e. mechanisms of regulation of smooth muscle tone) compared with resistance vessels (Busse *et al.*, 2000; Feletou & Vanhoutte, 1996; Rowell, 1993).

3.2.2 Apparent eNOS activity of isolated carotid and iliac arteries

In order to determine whether the *in vivo* observations regarding altered control of vascular tone by the L-arginine/nitric oxide pathway in cephalic and caudal vascular beds could be explained by differences in nitric oxide-producing capacities (reflecting NOS enzyme expression levels and/or activity), electrochemical measurement of acetylcholine-induced nitric oxide production from isolated carotid and iliac arteries from HDT and control animals was performed. In addition, the concentration-response relationship between acetylcholine and nitric oxide production from isolated arteries was investigated in the presence and absence of the non-selective NOS inhibitor L-NAME and the selective iNOS inhibitor 1400W.

As shown in Figure 3-13, acetylcholine induced a concentration-dependent increase in nitric oxide concentration in the perfusate of isolated carotid and iliac arteries. The maximal increase in nitric oxide production was significantly decreased in carotid arteries isolated from HDT rats compared with control animals (19.5±0.47 vs. 15.4±0.36 nM, P=0.001; Figure 3-13*A*). Following a 15 min infusion with 1400W, nitric oxide production remained unchanged

relative to the maximal levels prior to exposure to the inhibitor, however a significant difference between control and HDT groups was still observed (19.3±0.65 vs. 16.7±0.51 nM, P=0.008; Figure 3-13*A*). Infusion with L-NAME significantly decreased maximal acetylcholine-induced nitric oxide production in both control (7.9±0.40 vs. 19.5±0.47 nM; P<0.001) and HDT (7.3±0.8 vs. 15.4±0.36; P<0.001) groups, however comparison of maximal nitric oxide production between groups following L-NAME infusion determined no significant difference. No significant difference in EC_{50} values were determined between groups or in the presence or absence of either 1400W or L-NAME.

A similar concentration-dependent increase in nitric oxide production was observed in isolated iliac arteries (Figure 3-13*B*), however no significant difference between the maximal nitric oxide production was observed between arteries from control (17.7±0.67 nM) and HDT animals (16.3±0.44 nM). Infusion with 1400W had no effect on maximal nitric oxide production from iliac arteries from either experimental group, while L-NAME infusion significantly decreased maximal production from arteries isolated from control (7.1±0.9 vs. 17.7±0.67 nM) and HDT (8.5±0.60 vs. 16.3±0.44 nM) animals, albeit to a similar (non-significant) degree. No significant differences in EC_{50} values were determined between groups or in the presence or absence of either 1400W or L-NAME.

These data suggest that eNOS activity in carotid arteries is decreased following exposure to simulated microgravity, whereas eNOS activity in iliac arteries remains unchanged. Furthermore, the lack of effect of 1400W on acetylcholine-induced nitric oxide production suggests that iNOS makes a negligible or no contribution to nitric oxide production in either the carotid or iliac arteries. These findings are in partial agreement with the earlier *in vivo* findings suggesting decreased contribution of eNOS to vascular tone in cephalic vascular beds, but does

not account for the functional evidence suggesting increased contribution of eNOS in caudal vascular beds following simulated microgravity.

3.3 BIOCHEMICAL ASSESSMENT OF NITRIC OXIDE SYNTHASE EXPRESSION

3.3.1 Semi-quantification of arterial eNOS & iNOS protein expression

In light of the findings from both *in vivo* and *in vitro* experiments suggesting changes in the contribution of NOS isoforms to vascular tone in the cephalic and caudal vasculatures, semi-quantification of eNOS/iNOS protein levels in carotid, mesenteric and iliac artery homogenates from control or HDT rats was performed using western blotting.

Relative eNOS/iNOS protein expression was quantified as a ratio of the band density compared with that of β-actin. Figure 3-14 shows an example western blot from which such band densities were determined. Bands corresponding to eNOS (molecular weight ~135 kDa) and iNOS (molecular weight ~130 kDa) were identified between the 150 and 100 kDa molecular weight markers (lane M; Figure 3-14*A&B*). As shown in Figure 3-15*A*, eNOS expression was slightly higher in mesenteric arteries compared with carotid or iliac arteries (which exhibited similar levels of expression), however no significant difference in eNOS expression between control and HDT animals was determined in any of the arteries investigated. Figure 3-15*B* demonstrates that a small degree of basal iNOS expression was observed in all three arteries evaluated, however no significant difference between arteries from control or HDT animals was observed.

The findings from this study suggest that the differences in the contribution of NOS isoforms to vascular tone of cephalic and caudal vascular beds between control and HDT observed from *in vivo* and *in vitro* functional studies are likely not due to changes in levels of protein expression, but may reflect changes in enzyme activity.

3.3.2 Immunostaining of arterial eNOS & iNOS

In addition to semi-quantification of eNOS/iNOS protein expression levels using western blotting, immunohistochemical staining of the two NOS isoenzymes was performed on carotid, mesenteric and iliac arteries obtained from control and 14 day HDT rats. In contrast to western blotting, immunohistochemistry allows only qualitative evaluation of differences in endothelial protein levels, but with the advantage of having some degree of spatial resolution. Figure 3-16 shows example photographs of arterial sections stained for eNOS. Qualitative evaluation does not suggest any detectable significant differences in the immunostaining for eNOS between arteries from control and HDT rats. Staining for iNOS in the same arteries was negligible, and was difficult to distinguish from non-specific background staining. In light of the western blot data, at the resolution of microscopy used in this study it appears that 14 day HDT does not induce any significant changes in eNOS or iNOS expression in the three arteries investigated.

The data from this study suggest that the *in vivo* functional changes associated with alterations in the control of vascular tone by eNOS observed following simulated microgravity are likely not associated with changes in NOS protein expression.

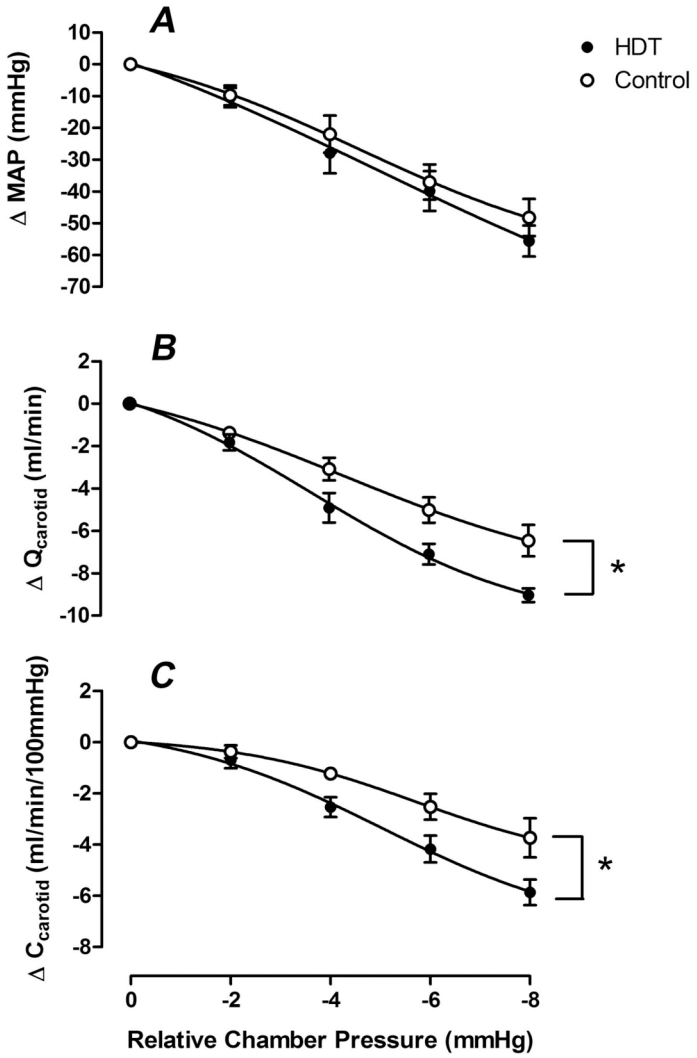

Figure 3-1 – Haemodynamic response to lower body negative pressure

Relationship between applied lower body negative pressure (LBNP) and mean arterial pressure (MAP; ***A***), carotid artery flow ($Q_{carotid}$; ***B***) and carotid artery conductance ($C_{carotid}$; ***C***) in thiobutabarbital-anaesthetised control (○) and 14 days of head-down tilt (HDT) rats (●). Data are displayed as the mean±SE change from baseline (n=8 per group). * denotes $P<0.05$ for two-way repeated-measures ANOVA.

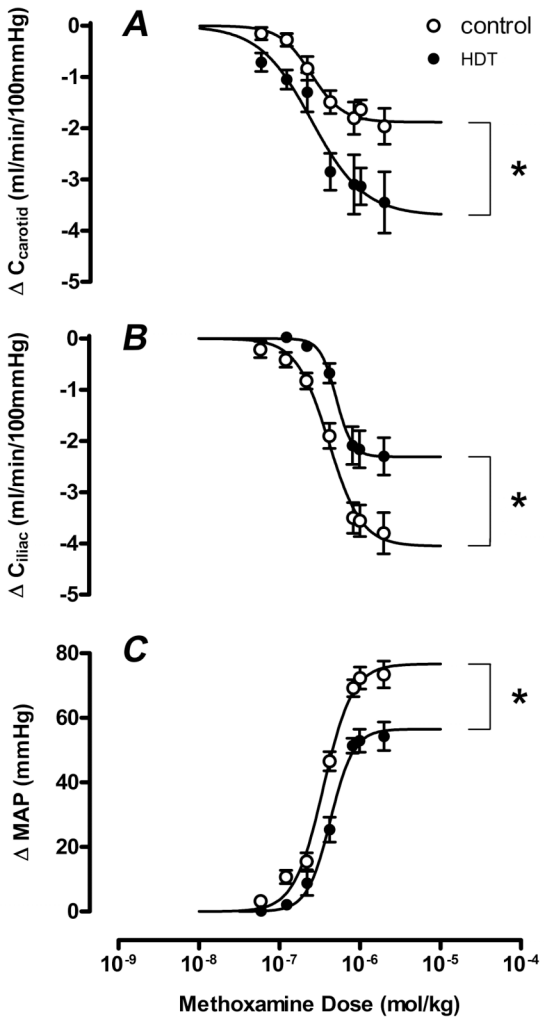

Figure 3-2 – Haemodynamic response to methoxamine

Dose-response relationships between methoxamine (i.v. bolus) and carotid artery conductance ($C_{carotid}$; ***A***), iliac artery conductance (C_{iliac}; ***B***) and mean arterial pressure (MAP; ***C***) in thiobutabarbital-anaesthetised control (○) and 14 day head-down tilt (HDT) rats (●). Data are displayed as the mean±SE change from baseline (n=7 per group). * denotes $P<0.05$ for two-way repeated-measures ANOVA followed by planned Bonferroni post-tests for differences in E_{max} values.

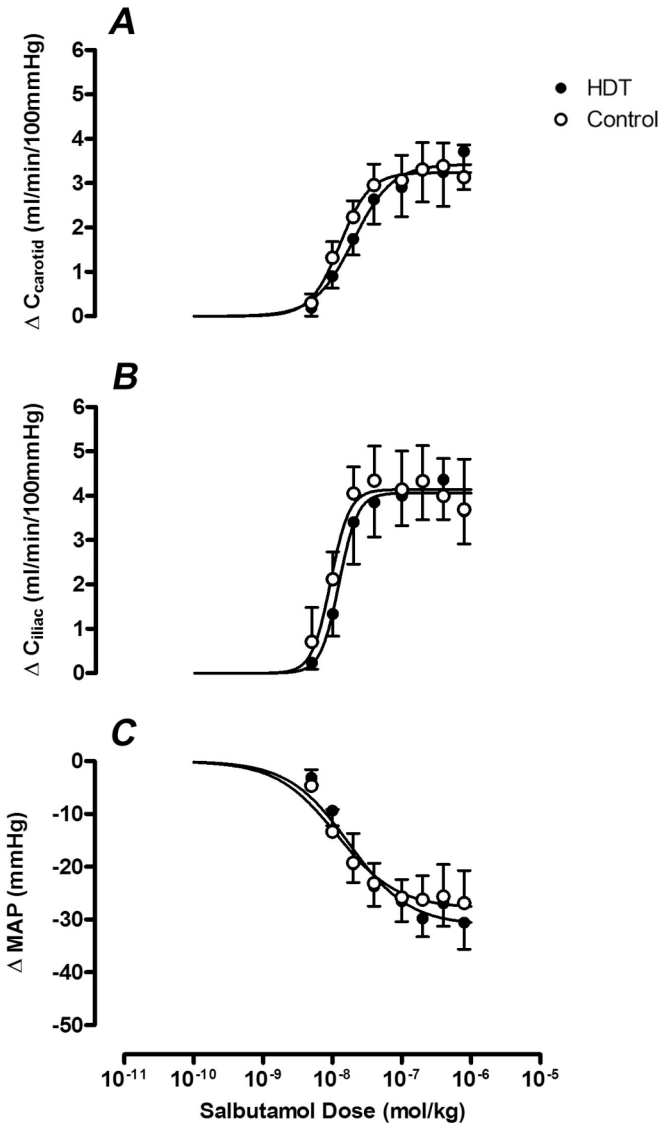

Figure 3-3 – Haemodynamic response to salbutamol

Dose-response relationship between salbutamol (i.v. bolus) and carotid artery conductance ($C_{carotid}$; ***A***), iliac artery conductance (C_{iliac}; ***B***) and mean arterial pressure (MAP; ***C***) in thiobutabarbital-anaesthetised control (○) and 14 day head-down tilt (HDT) rats (●). Data are displayed as the mean±SE change from baseline (n=7 per group). Two-way ANOVA determined no significant difference between E_{max} values for control and HDT groups for any variable measured.

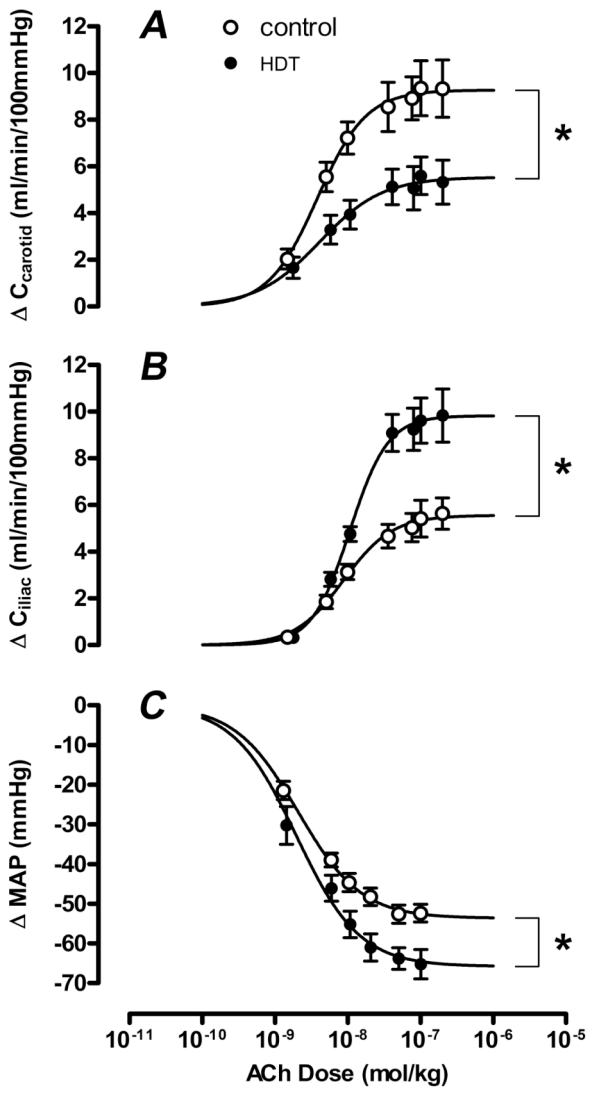

Figure 3-4 – Haemodynamic response to acetylcholine

Dose-response relationships between acetylcholine (ACh; i.v. bolus) and carotid artery conductance ($C_{carotid}$; ***A***), iliac artery conductance (C_{iliac}; ***B***) and mean arterial pressure (MAP; ***C***) in thiobutabarbital-anaesthetised control (○) and 14 day head-down tilt (HDT) rats (●). Data are displayed as the mean±SE change from baseline (n=7 per group). * denotes $P<0.05$ for two-way repeated-measures ANOVA followed by planned Bonferroni post-tests for differences in E_{max} values.

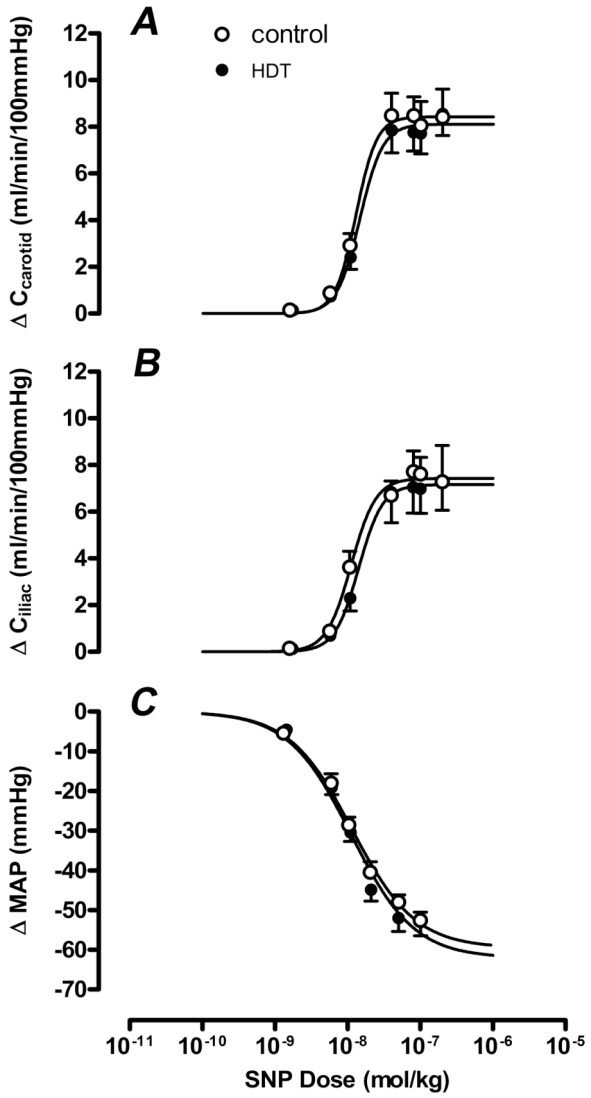

Figure 3-5 – Haemodynamic response to sodium nitroprusside

Dose-response relationships between sodium nitroprusside (SNP; i.v. bolus) and carotid artery conductance ($C_{carotid}$; ***A***), iliac artery conductance (C_{iliac}; ***B***) and mean arterial pressure (MAP; ***C***) in thiobutabarbital-anaesthetised control (○) and 14 day head-down tilt (HDT) rats (●). Data are displayed as the mean±SE change from baseline (n=7 per group).

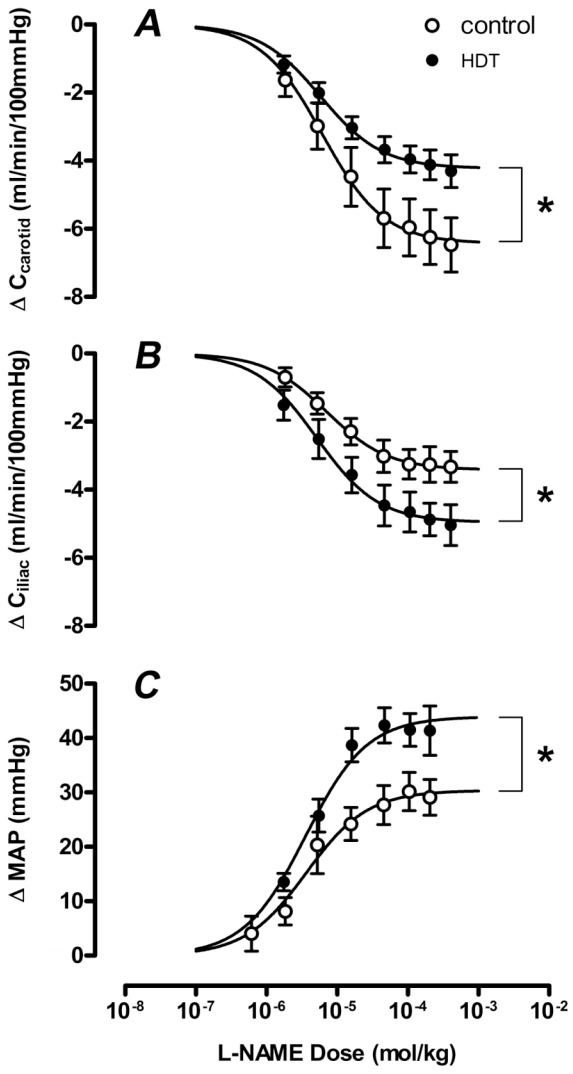

Figure 3-6 – Haemodynamic response to L-NAME

Dose-response relationships between N^{ω}-nitro-L-arginine methyl ester (L-NAME; i.v. bolus) and carotid artery conductance ($C_{carotid}$; ***A***), iliac artery conductance (C_{iliac}; ***B***) and mean arterial pressure (MAP; ***C***) in thiobutabarbital-anaesthetised control (○) and 14 day head-down tilt (HDT) rats (●). Data are displayed as the mean±SE change from baseline (n=7 per group). * denotes $P<0.05$ for two-way repeated-measures ANOVA followed by planned Bonferroni post-tests for differences in E_{max} values.

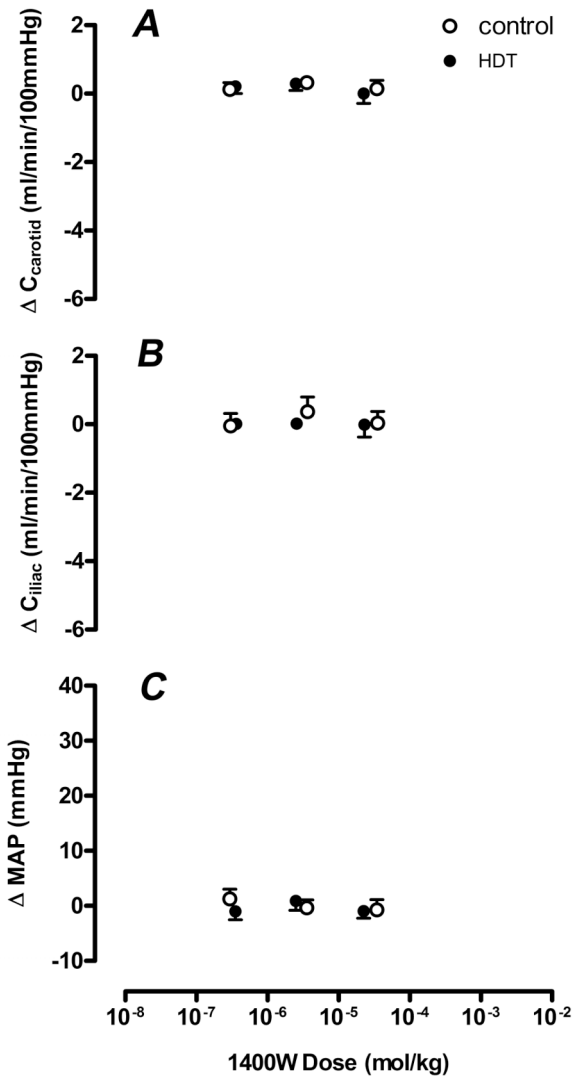

Figure 3-7 – Haemodynamic response to 1400W

Dose-response relationships between 1400W (i.v. bolus) and carotid artery conductance ($C_{carotid}$; ***A***), iliac artery conductance (C_{iliac}; ***B***) and mean arterial pressure (MAP; ***C***) in thiobutabarbital-anaesthetised control (○) and 14 day head-down tilt (HDT) rats (●). Data are displayed as the mean±SE change from baseline (n=7 per group). * denotes $P<0.05$ for two-way repeated-measures ANOVA followed by planned Bonferroni post-tests for differences in E_{max} values.

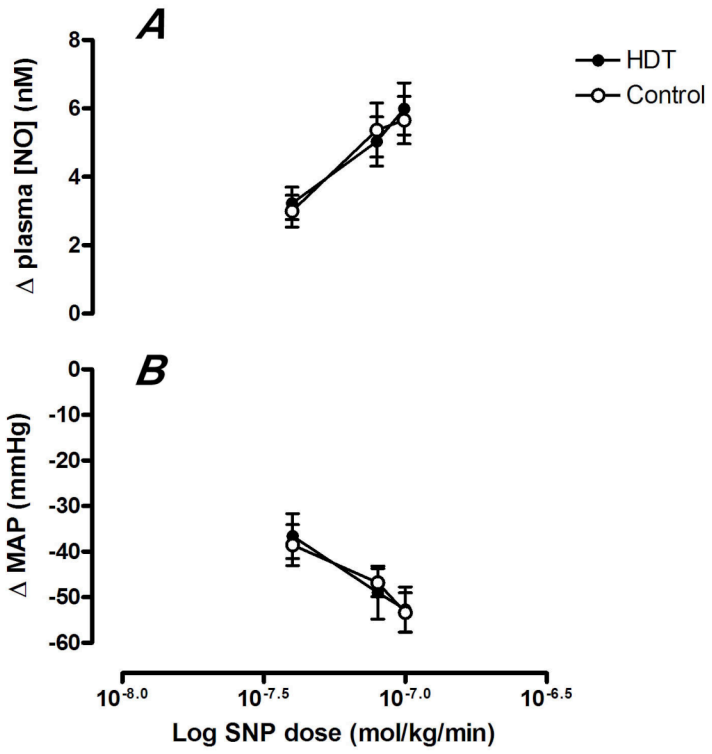

Figure 3-8 – Peak plasma [NO] and MAP response to sodium nitroprusside

Increase in plasma nitric oxide (NO) concentration (***A***) and mean depressor response (***B***) in response to intravenous sodium nitroprusside (SNP) infusion in control (○) and 14 day head-down tilt (HDT) rats (●). Data are expressed as the mean±SE change from baseline plasma NO concentration and mean arterial pressure (MAP) (n=7 per group). Two-way repeated measures ANOVA determined no significant difference between control and HDT groups for either variable.

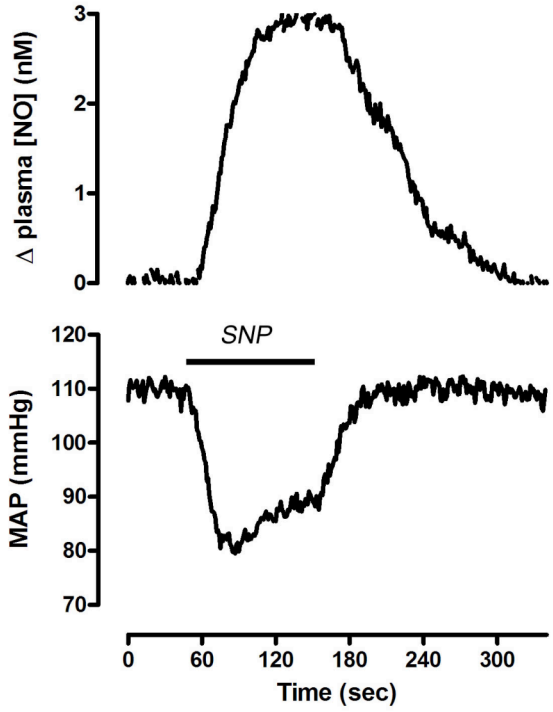

Figure 3-9 – Simultaneous recording of plasma [NO] and mean arterial pressure

Example trace displaying simultaneous measurement of plasma nitric oxide (NO) concentration and mean arterial pressure (MAP) in response to an intravenous infusion of sodium nitroprusside (SNP) at 40 nmol/kg/min in a thiobutabarbital-anaesthetised control animal. Plasma NO concentration was measured using an amperometric NO electrode located in the abdominal aorta calibrated using a saturated NO gas solution.

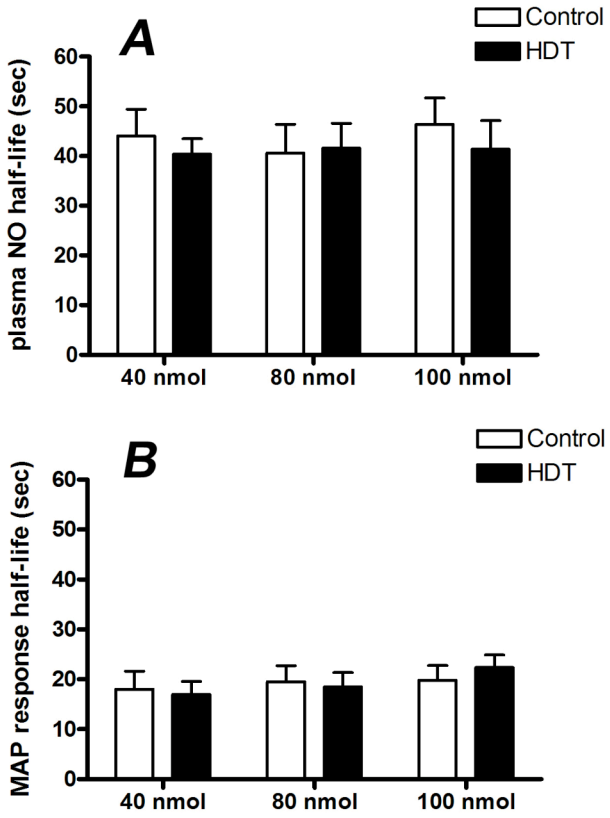

Figure 3-10 – Pharmacokinetic & pharmacodynamic half lives of NO

Mean half-life values for plasma nitric oxide (NO) concentration and for the mean arterial pressure (MAP) response to intravenous sodium nitroprusside (SNP) infusion at 40, 80 and 100 nmol/kg/min in control (open bars) and 14-day head down tilt (HDT) animals (solid bars). Data are expressed as the mean±SE half-life values determined from fitting a single exponential decay function to the raw plasma NO concentration and mean arterial pressure (MAP) data following cessation of SNP infusion (n=7 per group). Two-way repeated measures ANOVA determined no significant difference between control and HDT groups for either variable.

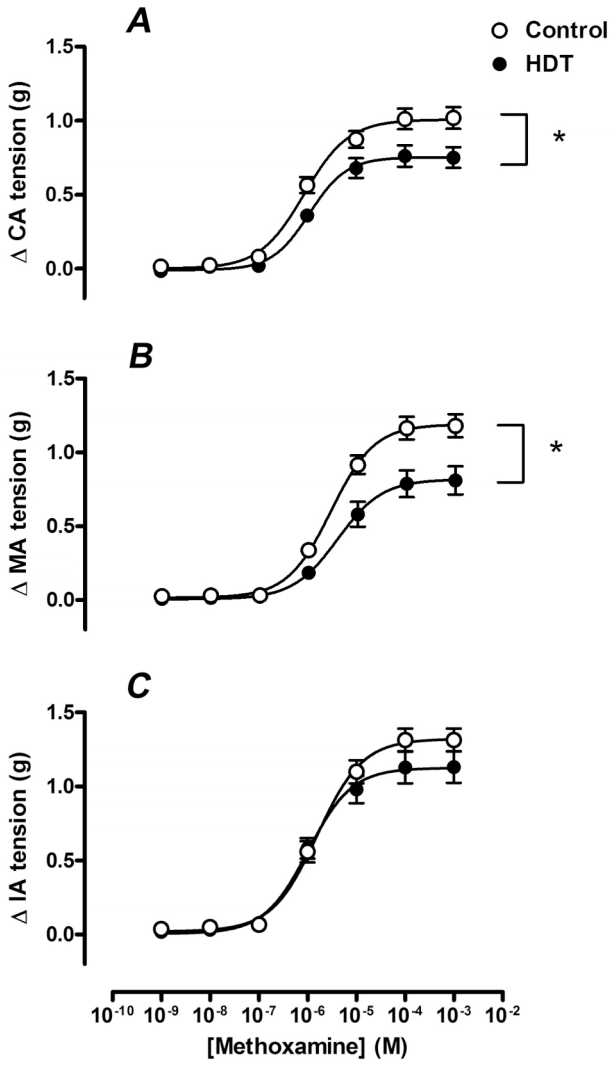

Figure 3-11 – Isometric contractile response of isolated arteries to methoxamine

Concentration-response curves (CRCs) for isolated carotid (***A***), mesenteric (***B***) and iliac (***C***) isometric contractile responses to methoxamine in control (○) and 14 day head-down tilt (HDT) rats (●). Data are displayed as the mean±SE change from baseline (***A***, ***B***, ***C***) or receptor occupancy (***D***, ***E***, ***F***) and represent n=7 per group. * denotes $P<0.05$ for two-way ANOVA followed by planned Bonferroni post-tests for differences in E_{max} values.

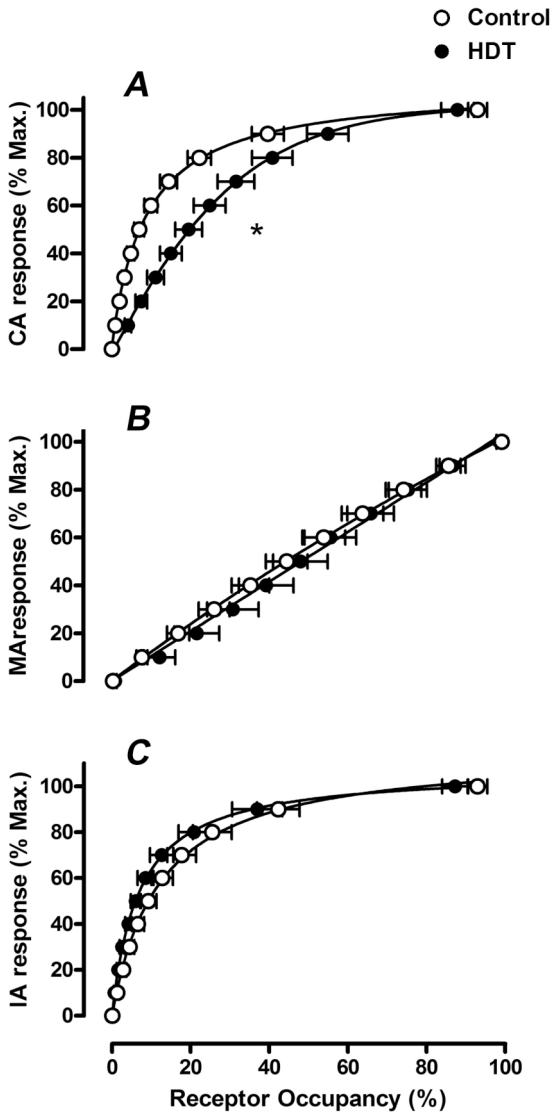

Figure 3-12 – Receptor occupancy-response relationships for isolated arteries

Receptor occupancy-response curves for isolated carotid (***A***), mesenteric (***B***) and iliac (***C***) calculated from the attenuation of isometric contractile responses to methoxamine following incubation with phenoxybenzamine (3 nM) for 5 minutes (using Furchgott's method) in control (○) and 14 day head-down tilt (HDT) rats (●). Data are displayed as the mean±SE receptor occupancy and represent n=7 per group. * denotes $P<0.05$ for two-way ANOVA followed by planned Bonferroni post-tests for differences in the receptor occupancy required for 50% of maximal response.

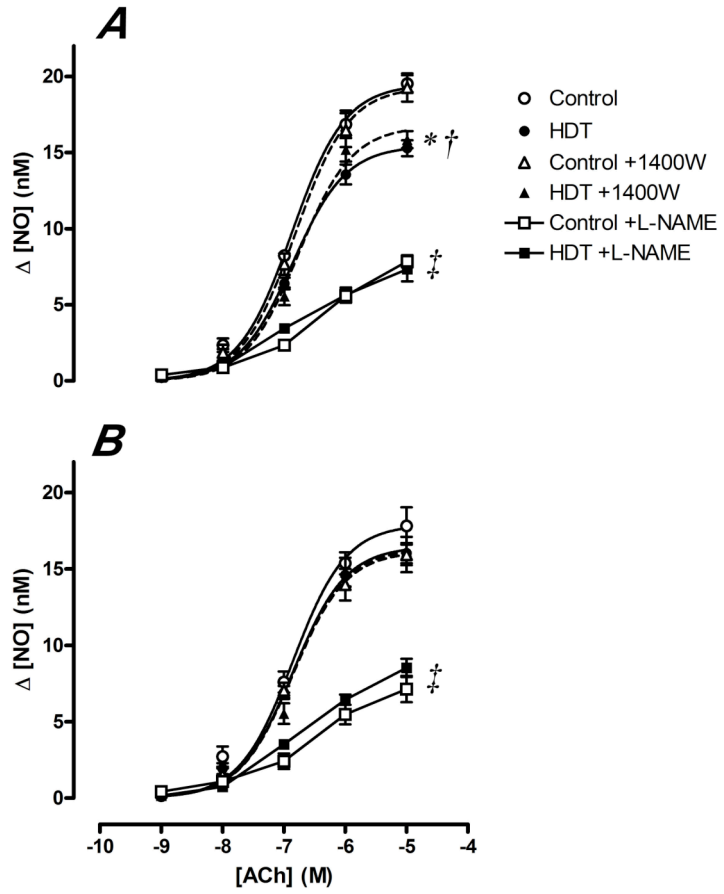

Figure 3-13 – NO-producing capacity of perfused isolated carotid & iliac arteries

Acetylcholine (ACh)-induced nitric oxide (NO)-release from isolated perfused carotid (***A***) and iliac (***B***) arteries. ACh was infused into the system at concentrations (in the perfusate) of 1 nM – 10 μM. Concentration-response curves (CRC) were generated to ACh in the absence or any inhibitors, and then following a 15 min infusion with either 1400W (2.5 μM) or L-NAME (20 μM). Data are expressed as the mean±SE change from baseline NO concentration (n=7 per group). * denotes $P<0.05$ for Two-way ANOVA followed by planned Bonferroni post-tests for differences in E_{max} values between groups in the absence of inhibitors or following 1400W (†). Two-way repeated measures ANOVA determined a significant difference between the CRC before and after L-NAME for both groups (‡), but no difference was observed when the responses between groups were compared.

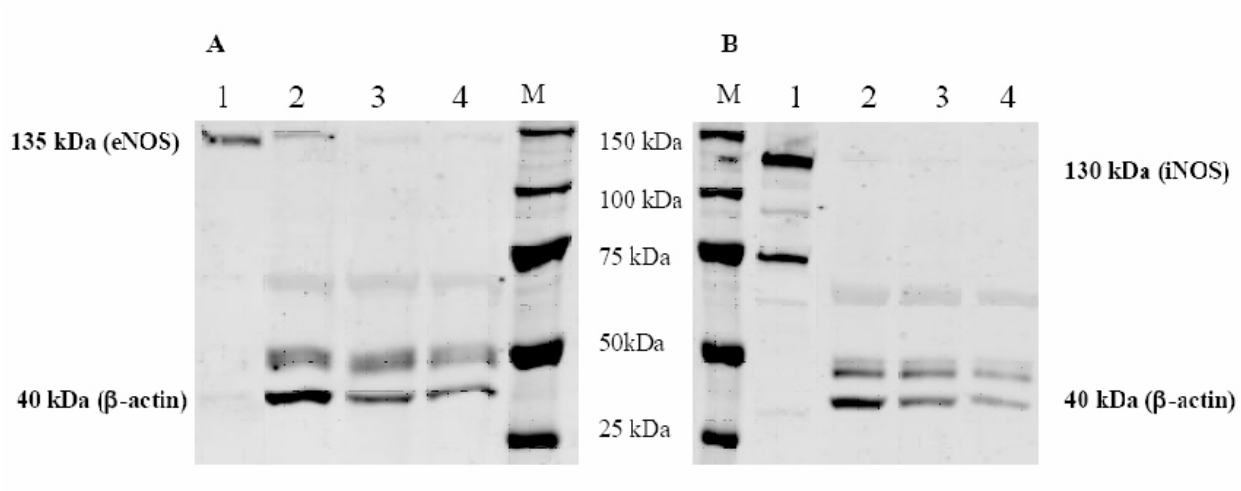

Figure 3-14 - Western blot for eNOS and iNOS

Example western blot for endothelial nitric oxide synthase (eNOS; **A**) and inducible nitric oxide synthase (iNOS; **B**) using tissue homogenates obtained from carotid (lane 2), iliac (lane 3) and mesenteric (lane 4) arteries from a control animal. Lane 1 contained recombinant eNOS or iNOS gel electrophoresis standards, lane M contained molecular weight markers. *β*-actin was employed as a control for equal lane loading; *β*-actin and eNOS/iNOS bands were determined using either primary mouse anti-*β*-actin, anti-eNOS or anti-iNOS monoclonal antibody detected with goat-anti mouse IgG secondary antibody with infrared fluorescent conjugate.

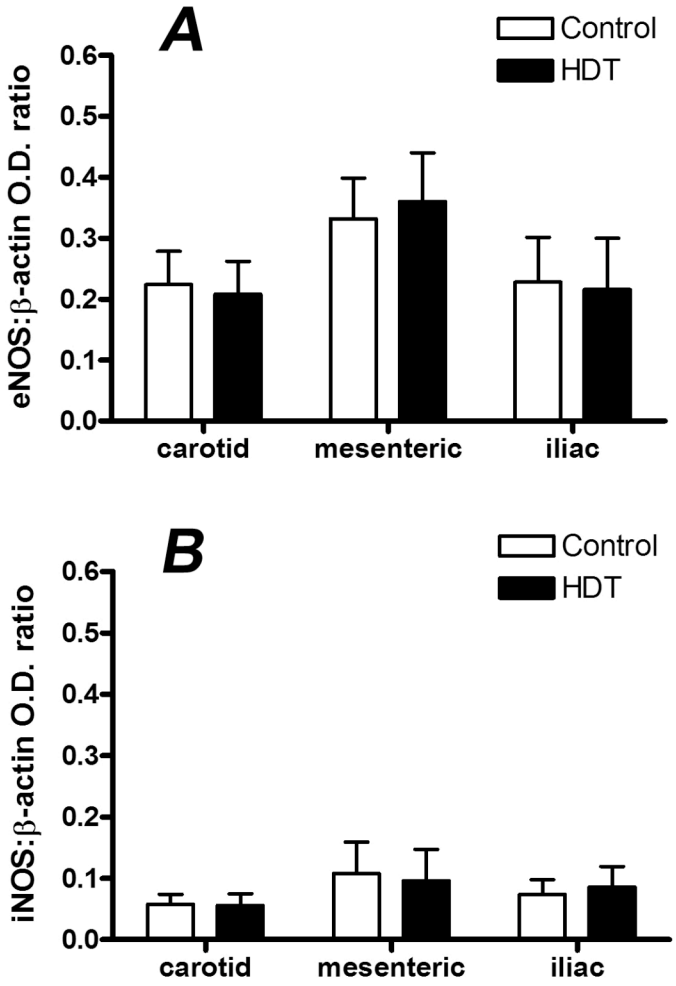

Figure 3-15 - eNOS & iNOS protein expression levels

Relative protein expression of eNOS and iNOS in carotid, mesenteric and iliac arteries from control (open bars) and 14-day HDT rats (solid bars). Data are displayed as the mean±SE optical density (O.D.) ratio between either eNOS or iNOS and β-actin for each lane analysed (n=5 animals per group). Two-way ANOVA revealed no significant difference between eNOS or iNOS expression in each artery between control and HDT groups when $\alpha = 0.05$.

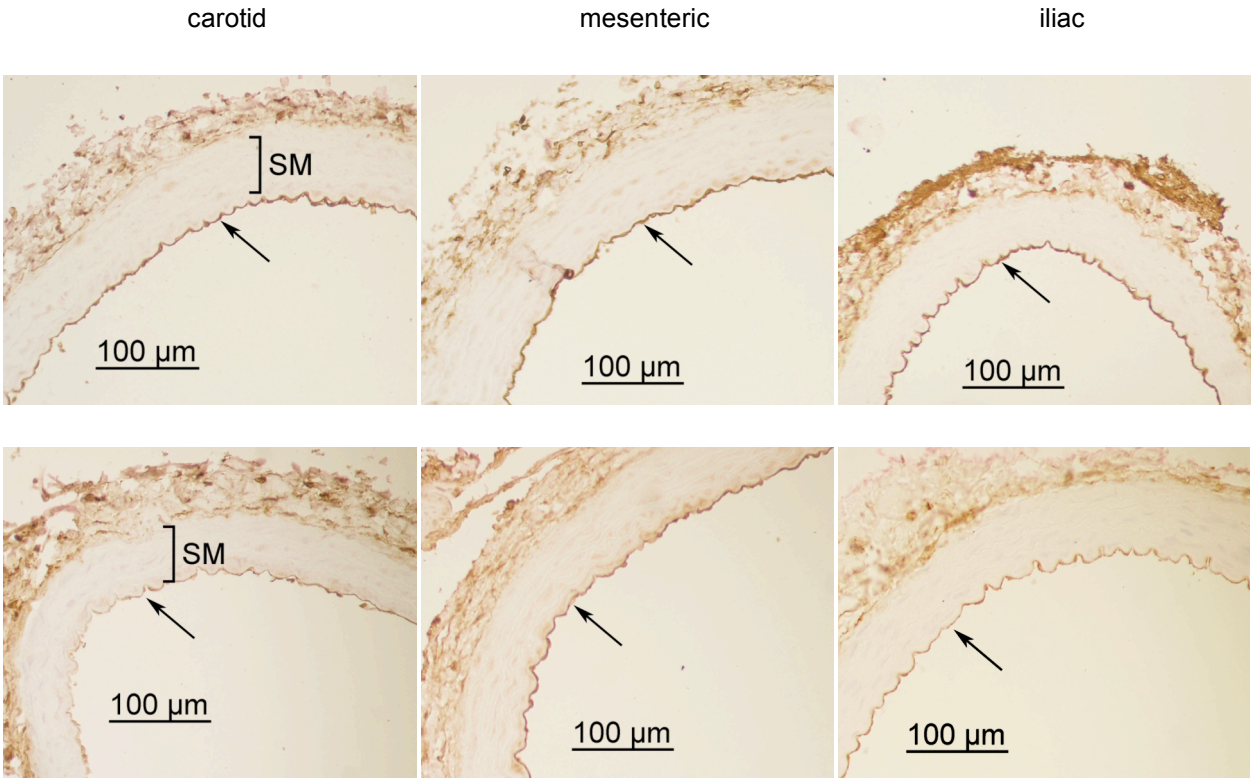

Figure 3-16 – Immunostaining of eNOS in carotid, mesenteric and iliac arteries

Example photographs showing immunostaining of endothelial nitric oxide synthase (eNOS; dark brown) in transverse slices of carotid, mesenteric and iliac arteries from control (upper panels) and 14 day head-down tilt (HDT) rats (lower panels). Slices were stained using primary mouse anti-eNOS monoclonal antibody detected with goat-anti mouse IgG secondary antibody with horseradish peroxidase conjugate. SM; smooth muscle layer. Arrows indicate endothelial cell layer.

Table 3-1 – Baseline cardiovascular parameters in control & 14 day HDT rats

	Protocol I		*Protocol II*		*Protocol III*	
	control	*HDT*	*control*	*HDT*	*control*	*HDT*
HR (beats/min)	371±10	362±11	369±7	354±11	364±7	362±9
MAP (mmHg)	106±6	99±4	103±3	109±4	101±4	102±4
$Q_{carotid}$ (ml/min)	11.0±0.6	10.6±0.7	11.1±0.7	10.0±0.6	*n/a*	*n/a*
Q_{iliac} (ml/min)	*n/a*	*n/a*	7.9±0.5	7.4±0.9	*n/a*	*n/a*
$C_{carotid}$ (ml/min/100mmHg)	9.9±1.0	10.5±0.7	10.8±0.7	10.0±0.7	*n/a*	*n/a*
C_{iliac} (ml/min/100mmHg)	*n/a*	*n/a*	7.8±0.6	7.0±0.4	*n/a*	*n/a*

Data are displayed as the mean±SE. HDT, 14-day head-down tilt; HR, heart rate; MAP, mean arterial pressure; $Q_{carotid}$, carotid artery blood flow; Q_{iliac}, iliac artery blood flow; $C_{carotid}$, carotid artery conductance; C_{iliac}, iliac artery conductance. *n/a* denotes that this cardiovascular parameter was not measured as part of this experimental protocol. Students *t*-test with Welch's correction for unequal variances revealed no significant differences in any of the variables measured between control and HDT rats for all three protocols.

Table 3-2 – Maximal drug-induced changes in regional flow in control or 14 day HDT rats

	ΔQ_{iliac} *(ml/min)*		$\Delta Q_{carotid}$ *(ml/min)*	
	control	*HDT*	*control*	*HDT*
methoxamine	-3.1±0.5	-1.3±0.4*	-1.4±0.3	-2.1±0.4
acetylcholine	-3.0±0.4	-6.5±0.7*	-4.9±0.6	3.6±0.5
Sodium nitroprusside	-4.4±0.6	-4.4±0.5	-5.0±0.5	-5.1±0.6
L-NAME	-1.0±0.3	-2.2±0.6	-2.0±0.4	1.8±0.3
1400W	*n/a*	*n/a*	*n/a*	*n/a*
salbutamol	-1.2±0.3	-1.3±0.4	-0.9±0.3	-1.1±0.4

Data are displayed as the maximum change from baseline of blood flow to the vasculature distal to the common iliac artery (ΔQ_{iliac}) or blood flow to the vasculature distal to the common carotid artery ($\Delta Q_{carotid}$) observed for each drug and represent the mean±SE; n=7 per group. HDT, 14-day head-down tilt; ACh, acetylcholine; SNP, sodium nitroprusside; L-NAME, N^{ω}-nitro-L-arginine methyl ester. * denotes $P<0.05$ for students *t*-test with Welch's correction for unequal variances for differences in ΔQ_{iliac} or $\Delta Q_{carotid}$ between control and HDT rats.

Table 3-3 - Fraction of remaining functional α_1-adrenoceptors and determined dissociation constants for methoxamine following incubation with phenoxybenzamine

	q		pK_A	
	control	*HDT*	*control*	*HDT*
Carotid artery	0.23±0.04	0.37±0.06	4.9±0.2	5.2±0.1
Mesenteric artery	0.63±0.09	0.47±0.04	5.4±0.2	5.3±0.1
Iliac artery	0.22±0.02	0.22±0.06	4.8±0.1	4.9±0.1

Data are displayed as the mean±SE; n=5 per group. q, fraction of functional α_1-adrenoceptors remaining in isolated arteries following incubation with 3 nM phenoxybenzamine for 5 min; pKA, determined dissociation constant for methoxamine; HDT, 14-day head-down tilt.

Table 3-4 - α_1-adrenoceptor reserve in isolated arteries from control and 14 day HDT animals

	α_1-adrenoceptor reserve	
	control	*HDT*
Carotid artery	15.2±3.2	6.4±1.7*
Mesenteric artery	2.4±0.4	2.8±0.3
Iliac artery	14.6±2.9	20.3±4.5

Data are displayed as the mean±SE; n=5 per group. HDT, 14-day head-down tilt.

4 DISCUSSION

4.1 REVIEW OF HYPOTHESES & SPECIFIC RESEARCH OBJECTIVES

4.1.1 Characterisation of the HDT model & demonstration of differential adaptation *in vivo*

Before the mechanisms underlying differential adaptation to microgravity could be investigated *in vivo*, demonstration of functional adaptation analogous to that thought to be associated with orthostatic intolerance in humans was necessary. As observed in Figure 3-1*B&C*, the cerebral haemodynamic response to LBNP was impaired following 14 days of HDT, with a greater decrease in $Q_{carotid}$ and $C_{carotid}$ observed in the HDT group compared with control. Moreover, this effect was not associated with any change in the hypotensive response (Figure 3-1*A*) or with a change in total blood volume (Section 3.1.1). As discussed in the *Introduction*, orthostatic intolerance following microgravity exposure may, or may not be associated with hypotension (Buckey *et al.*, 1996; Pavy-Le Traon *et al.*, 1995). While a decrease in MAP in response to LBNP was observed in this study, the lack of hypotensive effect between groups suggests that the impaired $C_{carotid}$ reflects alterations in cerebrovascular autoregulation of flow in response to hypotension (lower end of the flow-pressure curve; Figure 1-4). Whether the lack of difference in the hypotensive effect is due to the absence of hypovolaemia or impairment of the mechanisms controlling cardiac output and/or TPR in HDT relative to control animals is unknown. A negligible effect of HDT on blood volume has been observed previously (Chew & Segal, 1997), but has also been shown to vary between strains of animals used and the duration of HDT (Dunn *et al.*, 1985).

While clear effects on the cerebral haemodynamic response to LBNP were observed following HDT, demonstration of differential adaptation from the simultaneous measurement of

C_{iliac} (in addition to $C_{carotid}$) in response to LBNP was not possible due to technical limitations associated with the required longitudinal medial laparotomy for ultrasonic measurement of Q_{iliac}. Differential adaptation was however demonstrated *in vivo* from the cephalic, peripheral and caudal haemodynamic response to the α_1-adrenoceptor agonist methoxamine. In addition to a decreased pressor response (Figure 3-2*C*) similar to that previously reported following HDT (Sangha *et al.*, 2000), the vasoconstrictor response to methoxamine increased in the cerebral (cephalic) vascular bed, but decreased in the caudal vasculature (Figure 3-2*A&B*). These findings allow the hypothesis associated with research objective A to be accepted, and for the first time demonstrate differential adaptation of α_1-adrenoceptor-mediated vasoconstriction of cephalic and caudal vascular beds *in vivo*, in accordance with what might be predicted from previous *in vitro* studies (Delp *et al.*, 1993; Sangha *et al.*, 2000; Zhang, 2001). Perhaps not surprisingly, functional adaptation of β_2-mediated vasodilatation was not observed *in vivo* following HDT (Figure 3-3), in agreement with previous *in vitro* findings suggesting that adaptation of vascular α_1-adrenoceptors was not associated with any corresponding changes to vasodilator responses to catecholamines (Fadiukova *et al.*, 2005; Ma *et al.*, 1998b; McCurdy *et al.*, 2000).

The nature of differential adaptation of α_1-adrenoceptor function following HDT was investigated further by assessing vasoconstrictor responses and α_1-adrenoceptor reserve in isolated carotid, mesenteric and iliac arteries *in vitro*. Maximal contractions to methoxamine were attenuated in carotid and mesenteric arteries, but not iliac arteries, isolated from HDT rats compared to control (Figure 3-11). These differences were associated with a decrease in α_1-adrenoceptor reserve in carotid arteries, but not mesenteric (Figure 3-12; Table 3-4). These findings are in partial agreement with previous studies reporting reduced α_1-adrenoceptor-mediated contraction of various conduit (carotid/mesenteric/femoral) arteries following HDT

(Delp *et al.*, 1993; Purdy *et al.*, 1997; Sangha *et al.*, 1999), and are the first to demonstrate that such differences may be associated with decreases in α_1-adrenoceptor reserve. However, while α_1-adrenoceptor reserve was reduced in isolated carotid arteries following HDT, a significant degree of receptor reserve (6.4±1.7) was still observed (Table 3-4), meaning that the same E_{max} observed in control animals should have been attainable (Furchgott, 1966; Purdy & Stupecky, 1984). The significance of any observed differences in receptor reserve between control and experimental animals may be understood from the concept that efficacy (*E*; defined by Stephenson as a measure of the capacity of an agonist to initiate a response) is related to the intrinsic efficacy of an agonist (*e*) and the total concentration of active receptors (R_t) by equation 9 (Stephenson, 1956):

$$E = e \bullet R_t \qquad \text{(Eq.9)}$$

The research presented above regarding α_1-adrenoceptor reserve employs the same method as described by Purdy and Stupecky (Purdy & Stupecky, 1984), where 'receptor reserve' and 'efficacy' are considered interchangeable (functionally defined as the reciprocal of the fraction of receptors occupied by agonist at EC_{50}). While this method cannot distinguish between changes *e* and R_t, it is apparent that changes in receptor reserve – depending on the magnitude of such change – can have significant effects on α_1-adrenoceptor-mediated contractile function. Further investigation into the nature of the changes in α_1-adrenoceptor function (for example measuring receptor density *via* a radioligand binding assay) will certainly aid interpretation of the functional data obtained from this study.

The *in vitro* findings regarding α_1-adrenoceptor function (i.e. decreased constriction of isolated carotid artery rings) following simulated microgravity are obviously in contrast with the above *in vivo* observation of increased vasoconstriction in the cephalic arterial bed following

HDT. This disparity may potentially reflect differences in adaptation between conduit arteries such as the common carotid and smaller, more distal arteries and arterioles that may play a larger role in regulation of vascular resistance in the cerebral circulation, such as the basilar and middle cerebral arteries (Faraci, 1990). In this regard, further functional *in vitro* studies are warranted, perhaps employing wire or pressure myography techniques to determine whether α_1-adrenoceptor function and/or receptor reserve are altered in such vessels. Together with the use of radioligand binding/western blotting to quantify receptor expression and density, these studies would uncover whether the observed differential response to methoxamine *in vivo* truly reflects changes in α_1-adrenoceptor reserve or alterations in the balance between vasoconstrictor stimuli and/or responsiveness and vasodilator stimuli and/or responsiveness.

4.1.2 Differential adaptation to HDT is associated with functional changes to the L-arginine/nitric oxide pathway

Following demonstration of differential functional adaptation to HDT *in vivo*, the effects of various pharmacological agents known to interact with the L-arginine/nitric oxide pathway were investigated to determine if changes in the contribution of this pathway to vascular tone represents a potential mechanism for such adaptation. *In vivo* acetylcholine-induced endothelium-dependent vasodilatation was decreased in the cephalic vasculature, but increased in the peripheral and caudal vascular beds following HDT (Figure 3-4). Furthermore, *in vivo* endothelium-independent vasodilatation was unaltered in the same vascular beds, demonstrated by a lack of significant difference in the haemodynamic response to both salbutamol (in the presence of L-NAME; Figure 3-3) and SNP (Figure 3-5) between HDT and control groups. The effects of HDT on $C_{carotid}$ with acetylcholine and SNP are in agreement with a corresponding *in vitro* study by Prisby *et al.* (2006) demonstrating impaired endothelium-dependent vasodilatation of middle cerebral arteries from 14 day HDT rats, but no effect on endothelium-

independent vasodilatation to SNP (Prisby *et al.*, 2006). Acetylcholine can produce vasodilatation through several pathways other than nitric oxide release, including stimulating the release of EDHF and PGI_2 (Feletou & Vanhoutte, 1996; Rubanyi, 1991). As changes in both these pathways have been demonstrated following HDT (Eatman *et al.*, 2003a; Prisby *et al.*, 2006), inhibition of nitric oxide synthesis was utilised as a method for selectively investigating changes in the L-arginine/nitric oxide pathway in the aforementioned differential functional adaptation. Non-selective NOS inhibition using L-NAME (Figure 3-6) revealed that nitric oxide synthesis is differentially altered following exposure to HDT, with a decreased contribution of NOS to vascular tone in the cephalic vasculature accompanied by an increased contribution in peripheral and caudal vascular beds. Furthermore, the lack of response to the selective iNOS inhibitor 1400W (Figure 3-7) suggests that the observed differential adaptation of NOS following HDT is not associated with induction of iNOS.

Together, these data allow acceptance of the hypothesis associated with research objective B, demonstrating differential functional adaptation of the contribution of nitric oxide to vascular tone in cephalic versus peripheral/caudal vascular beds. Furthermore, the haemodynamic responses observed following administration of L-NAME, but not 1400W, suggest that increased synthesis of nitric oxide via either eNOS and/or nNOS likely underlies such adaptation. Decreased and increased vasodilator tone from the L-arginine/nitric oxide pathway in cephalic and peripheral/caudal vascular beds, respectively, provides a potential explanation for the discrepancy between *in vitro* and *in vivo* responses to methoxamine following HDT discussed above. Nitric oxide produced by eNOS contributes to the regulation of vascular tone by providing vasodilator stimuli, the proportion of this contribution varying between the location and size of artery (Busse *et al.*, 2000; Preckel *et al.*, 1996; Rees *et al.*, 1989). A reduction in the contribution of eNOS to cephalic vascular tone (as observed from the

decreased response of $C_{carotid}$ to acetylcholine and L-NAME) following simulated microgravity would likely result in an increased response to vasoconstrictor stimuli, as observed from the $C_{carotid}$ response to methoxamine in HDT rats.

Likewise, in the peripheral vasculature (such as the hind limb vascular bed) increases in eNOS-mediated vasodilator tone (as suggested by the increased C_{iliac} response to acetylcholine and L-NAME) would likely result in impaired responses to vasoconstrictor stimuli, as observed from the C_{iliac} response to methoxamine in HDT rats. The increased pressor response to L-NAME and increased depressor response to acetylcholine following HDT suggest that the total peripheral vasculature adapts to simulated microgravity in similar fashion to the hind limb vasculature i.e. increased contribution of eNOS to vascular tone.

As discussed in the *Introduction*, previous studies involving determination of the expression of iNOS mRNA and use of 'selective' inhibitors of iNOS (aminoguanidine/AMT) have suggested that induction of iNOS may account for the observed alterations in the L-arginine/nitric oxide pathway as a potential mechanism underlying the arterial adaptation to microgravity (Eatman *et al.*, 2003b; Sangha *et al.*, 2000; Vaziri *et al.*, 2000). The results from such studies are not in agreement with the present findings discussed above from the use of the highly selective iNOS inhibitor 1400W. As previously mentioned, the loss of selectivity for iNOS exhibited by both aminoguanidine and AMT when used *in vivo* (Garvey *et al.*, 1997; Laszlo *et al.*, 1995) limit their use in integrative pharmacological research. In contrast, 1400W - even when administered at doses (0.2- 20 μmol/kg) up to five-fold greater than that reported as sufficient (4 μmol/kg) to completely reverse the iNOS-mediated vascular effects in an *in vivo* rat model of LPS-induced endotoxaemia (Garvey *et al.*, 1997) - produced no observable effects on cephalic, peripheral or caudal vascular tone in control or HDT animals, as might be expected

from the negligible role iNOS is thought to play in the regulation of vascular tone under normal conditions (Busse *et al.*, 2000; Kadowitz & McNamara, 2000; Moncada & Higgs, 2006).

4.1.3 Differential adaptation of L-arginine/nitric oxide pathway is associated with changes in regulation of eNOS activity

The differential adaptation of the L-arginine/nitric oxide pathway suggested by the *in vivo* findings above may be a result of one or more changes to several aspects of the pathway, including altered levels of NOS enzyme expression, NOS enzyme activity, rate of removal of nitric oxide and the pharmacodynamics of sGC activation (Busse *et al.*, 2000; Moncada & Higgs, 2006). Biochemical measurements of eNOS and iNOS protein expression levels (Figure 3-15 & Figure 3-16) suggest that total levels of enzyme expression of either isoform remain unchanged in carotid, mesenteric and iliac arteries following HDT. Therefore, the observation that acetylcholine-induced nitric oxide production from isolated carotid arteries (Figure 3-13) is decreased following HDT likely reflects alterations in NOS activity in these vessels. Moreover, the lack of effect of 1400W on *in vitro* nitric oxide production further suggests that eNOS - not iNOS - is responsible for the observed differences between control and HDT rats. Interestingly, no difference in nitric oxide production *in vitro* between HDT and control rats was observed in isolated iliac arteries. This may reflect differences between the cephalic and caudal vascular beds with regards to the order/size of artery that is involved in the functional adaptation to HDT, as the *in vivo* conductance measurements represent the total vascular tone of the beds distal to the location where flow is being measured. Together, these findings are in contrast to both our hypothesis (Section 1.3.2*B*) proposing that changes in NOS expression are responsible for differential adaptation of the L-arginine/nitric oxide pathway to simulated microgravity, and previous findings suggesting that downregulation of eNOS contributes to enhanced vasoconstriction of isolated middle cerebral arteries (Wilkerson *et al.*, 2005).

An extension of the hypothesis regarding the level at which the L-arginine/nitric oxide pathway is altered in response to HDT was that the observed effects on the peripheral vasculature (i.e. increased pressor response to L-NAME and depressor response to acetylcholine) were not due to changes in the pharmacokinetic or pharmacodynamic half-lives of nitric oxide (reflecting the rate of removal of nitric oxide from the circulation and duration of action on cellular effector mechanisms, respectively). Using the non-enzymatic nitric oxide donor, SNP, simultaneous measurement of MAP and real-time amperometric measurement of plasma nitric oxide concentration demonstrated no difference in the pharmacokinetic or pharmacodynamic half life. In summary, evaluation of potential changes in the L-arginine/nitric oxide pathway that may account for differential adaptation following simulated microgravity suggest a possible role for regional variations in the regulation of eNOS activity.

As discussed in the *Introduction*, eNOS activity is considered to be primarily regulated not through changes in protein expression levels, but through post-translational modification (through myristoylation/palmitoylation) leading to altered trafficking to and interaction with caveolae (Busconi & Michel, 1993; Garcia-Cardena *et al.*, 1996; Liu *et al.*, 1995). From the above findings suggesting alterations in eNOS activity may contribute to differential adaptation following HDT, further investigation into how the various mechanisms regulating eNOS activity may be altered are necessary. One mechanism of eNOS regulation has been previously investigated in the HDT model; expression of the scaffolding protein caveolin-1 was found to be decreased in middle cerebral arteries following 14 days of HDT (Prisby *et al.*, 2006). Caveolin-1, in addition to being a key structural element and primary marker of caveolae in vascular smooth muscle and endothelial cells, binds to and negatively modulates eNOS in a competitive manner with calcium-bound calmodulin (Cohen *et al.*, 2004). It is therefore surprising that caveolin-1 expression was found to be down-regulated following HDT, as this is

counterintuitive to the findings from this thesis and previous studies suggesting decreased nitric oxide synthesis in the cerebrovasculature (Wilkerson *et al.*, 2005). A potential explanation offered by Prisby and colleagues is that the biology of caveolin-1 is more complex than simply providing structure to caveolae and inhibition of eNOS. Indeed, caveolin-1 has been shown to play a regulatory role in the contractility of vascular smooth muscle cells (Je *et al.*, 2004), and in smooth muscle proliferation (Peterson *et al.*, 2003). Such a role for caveolin-1 in smooth muscle proliferation may represent a possible mechanism by which the differential structural adaptation discussed in the *Introduction* occurs.

4.1.4 Possible future research directions related to the L-arginine/nitric oxide pathway

The research presented in this thesis represents what is considered by the author to be a valuable contribution to the understanding of changes in the L-arginine/nitric oxide pathway as a possible mechanism underlying differential arterial adaptation to simulated microgravity. However, as with most (if not all) doctoral theses, it is by no means considered exhaustive. Over the course of the project – including the preparation of this thesis – numerous potential avenues for future studies were identified. To add further relevance to the findings discussed thus far, such areas for possible expansion are worth considering. Changes in the regulation of NOS activity between cephalic and peripheral/caudal vascular beds is proposed as a mechanism for differential functional adaptation to simulated microgravity. While the major pathway of such regulation have been discussed, and even investigated following HDT in previous studies (Prisby *et al.*, 2006), several other, more subtle influences on eNOS activity warrant further study.

Substrate (L-arginine) and co-factor (flavin mononucleotide; flavin adenine dinucleotide; tetrahydrobiopterin) availability, while obviously being a major component of the capacity to produce nitric oxide are not usually considered primary determinants of eNOS activity due to

the fact that they are usually found in vast excess. For example, normal physiological intracellular concentrations of L-arginine have been found to be sufficient for complete saturation of eNOS substrate binding (Moncada *et al.*, 1989; Su *et al.*, 1997). However, more recently the importance of extracellular L-arginine concentrations has been suggested for 'optimal synthesis by eNOS and iNOS in the rat mesenteric artery' (MacKenzie & Wadsworth, 2003), meaning that plasma L-arginine concentrations may well play a part in the regulation of eNOS activity and therefore deserves attention in the context of vascular adaptation following HDT. Furthermore, studies in mice genetically modified to over-express eNOS have suggested that tetrahydrobiopterin availability may play a more important role in the regulation of NOS activity than earlier studies indicated (Bendall *et al.*, 2005).

The discovery of asymmetrical dimethylarginine (ADMA) as an endogenous inhibitor of NOS in the early 1990s added yet another mechanism by which eNOS activity is known to be regulated (Kimoto *et al.*, 1993; MacAllister *et al.*, 1994; Vallance *et al.*, 1992). Based on the measurement of relatively high concentrations of ADMA in brain tissue (Kimoto *et al.*, 1993; Kotani *et al.*, 1992) and the demonstration that it constricts cerebral arteries and can inhibit agonist-mediated vasodilatation *in vivo*, (Faraci *et al.*, 1995), a role for ADMA in the regulation of cerebral eNOS activity, and therefore cerebrovascular tone has been proposed. Therefore, in light of the observed changes in the L-arginine/nitric oxide pathway following HDT, investigation into how ADMA expression and function may be altered by simulated microgravity is considered necessary.

While the focus of this thesis has been adaptation of the arterial system in response to simulated microgravity, as mentioned in the *Introduction* section, adaptation of the venous system has been previously observed in response to both simulated and actual microgravity (Dunbar *et al.*, 2000; Kirsch *et al.*, 1984; Sayet *et al.*, 1995). Similar to the adaptations observed

in the arterial system discussed above, attenuated α-adrenoceptor-mediated decreases in venous compliance and venoconstriction of the vena cava and mesenteric small veins has been observed in rats following periods of HDT (Dunbar *et al.*, 2000; Dunbar *et al.*, 2001; Sayet *et al.*, 1995). Given that the L-arginine/nitric oxide pathway has been demonstrated to play a role in regulating venous tone in both humans and laboratory rodents (Blackman *et al.*, 2000; Glick *et al.*, 1993), investigation into potential changes in this pathway in the venous system in response to microgravity exposure are warranted.

The role of nNOS in the local control of vascular tone has long been considered negligible (Busse *et al.*, 2000; Moncada & Higgs, 2006), and presumably for this reason has received little attention in the area of arterial adaptation to microgravity. Whether this reflects a true deficit in attention from the scientific community involved in such research, or merely the convention of not publishing negative or 'neutral' findings is unclear. However, studies investigating nitric oxide mediated cerebral vasodilatation suggest nNOS plays a significant role in regulating vascular tone in discrete areas of the brain in response to hypoxia and volatile anaesthetics (Bauser-Heaton & Bohlen, 2007; Staunton *et al.*, 2000). Furthermore, and of particular relevance to this thesis, cerebral autoregulatory vasodilatation in response to hypotension is also thought to have a significant nNOS component (Bauser-Heaton & Bohlen, 2007).

As mentioned previously, other endothelium-derived vasodilator mediators have been implicated in the adaptation to HDT (Eatman *et al.*, 2003a; Prisby *et al.*, 2006; Sangha *et al.*, 2001). While some evidence exists suggesting changes in the contribution of PGI_2 to vascular tone following HDT, other studies have failed to find any differences in prostanoid-mediated vasodilatation (Schrage *et al.*, 2000). As discussed previously, EDHF is know to play a significant role in endothelium-dependent vasodilatation in the cerebral circulation, and it is

therefore surprising that this mediator has not received more attention from researchers in the microgravity area. At the time of writing, only one paper in the literature specifically addresses this pathway in the HDT model, demonstrating that EDHF-mediated vasodilatation of isolated rat middle cerebral artery is enhanced following 14 days of simulated microgravity (Prisby *et al.*, 2006). As 14 days of HDT is associated with a simultaneous decrease in NOS-mediated vasodilatation of middle cerebral arteries in the same study, Prisby and colleagues offer the hypothesis that EDHF upregulation is a compensatory response to the reduction in the contribution of nitric oxide to cerebrovascular tone. Indeed, such compensation between nitric oxide and EDHF has been observed in models of other pathological states, including reperfusion injury in stroke (Marrelli *et al.*, 1999) and severe traumatic brain injury (Golding *et al.*, 1998), implying that the relative balance between nitric oxide and EDHF in the control of vascular tone is of vital importance to normal function in the cerebral vasculature.

As described in the *Introduction*, one of the intracellular mechanisms by which nitric oxide can produce relaxation of vascular smooth muscle is *via* a direct interaction with Ca^{2+}-dependent K^+ channels (Bolotina *et al.*, 1994; Robertson *et al.*, 1993). To date only two studies have investigated the effect of simulated microgravity on smooth muscle K^+ channel function. Following 1 and 4 weeks of HDT, electrophysiological measurements performed by Fu and colleagues demonstrated a more depolarised membrane potential in isolated myocytes from middle cerebral arteries, which was associated with decreased K^+ current densities (Fu *et al.*, 2004b). Interestingly, the opposite (i.e. more hyperpolarised membrane potential associated with increased K^+ current densities) was found in isolated mesenteric artery myocytes. A further study from the same laboratory investigated which K^+ channel subtypes were responsible for the observed electrophysiological changes following 4 weeks of HDT (Xie *et al.*, 2005). The decreases in current densities in cerebrovascular myocytes observed in the previous study

were attributed to functional alterations (decreases) in voltage-gated K^+ channels, but not to changes in large conductance K^+ channels. Together, these data demonstrate that functional adaptation of the control of vascular smooth muscle cell membrane potential by K^+ channels may be differentially altered by exposure to microgravity. Further investigations into such adaptations and how they may affect the vascular L-arginine/nitric oxide pathway (including the use of subtype-selective K^+ channel blockers *in vivo*) are certainly justified.

Recently, an alternative theory has been proposed by Delp and co-workers to explain differential adaptation following exposure to microgravity (Colleran *et al.*, 2008). They suggest that local arterial responses to differences in transmural pressure exerted on the vasculature along the cephalocaudal axis may not be the primary mechanism by which vasoconstriction is increased/decreased, a concept suggested in several previous studies but largely ignored by the scientific community until recently (Overton *et al.*, 1989). Based on the repeated observation (both in their laboratory and others) that mesenteric artery contractile function is diminished following microgravity (despite being located at the level of the hydrostatic indifference point), a potential role for a circulating factor(s) has been suggested. Plasma concentrations of ANP and NH_2-terminal prohormone brain natriuretic peptide (BNP) have been observed to be increased following 14 days of HDT, leading to the authors' suggestion of ANP and BNP as candidates for such deconditioning circulating factors. In the same study, the demonstration that pre-incubation with either ANP or BNP was able to diminish contractile responses of isolated small mesenteric arteries and veins further supports this theory. Further investigation into potential cellular mechanisms underlying such deconditioning in mesenteric arteries by the same group suggests that decreased Ca^{2+}-induced Ca^{2+}-release as a result of decreased ryanodine 2 receptor protein expression may play an important role (Behnke *et al.*, 2008). While these novel theories require further investigation, they may explain why the systemic circulation largely

exhibits impaired vasoconstriction to catecholamines, while the cerebrovasculature - protected from circulating hormones by the blood brain barrier – is not subject to deconditioning, and can even show enhanced vasoconstriction.

4.2 SIGNIFICANCE OF FINDINGS

4.2.1 Implications for potential countermeasures

The findings from the research presented in this thesis provide evidence for a potential mechanism underlying differential arterial adaptation to microgravity involving altered eNOS activity. An increased contribution to peripheral and/or lower limb vascular tone by eNOS would potentially further impair the already compromised ability to raise TPR in response to orthostatic stress following spaceflight or HDBR, exacerbating any observed orthostatic hypotension. Moreover, decreased eNOS activity in the cerebral vasculature may result in inadequate cerebral autoregulatory vasodilatation in response to such hypotension, thereby compromising cerebral perfusion. Because of this bidirectional adaptation in the contribution of eNOS to vascular tone, pharmacological intervention with a selective inhibitor of eNOS may not necessarily be effective in the prevention of orthostatic intolerance following spaceflight; any benefit imparted from raising TPR or increasing venous return (and therefore cardiac output) could be outweighed by further impairment of cerebral autoregulatory vasodilatation.

This scenario may arise assuming that the eNOS isoform responsible for adaptation was the same in the cerebral and peripheral vasculature. Until recently, very little evidence existed suggesting discrete subpopulations of eNOS. This is in contrast to nNOS, for which four distinct splice variants have been identified in several tissues (Alderton *et al.*, 2001; Wang *et al.*, 1999). While three different splice variants of eNOS have recently been identified (Lorenz *et al.*, 2007), their role in the regulation of vascular eNOS activity is unknown, and splice variant-

specific inhibitors are yet to be developed. In light of the relatively few number of selective inhibitors of eNOS identified/developed to date, selective pharmacological modulation of cephalic and peripheral eNOS is currently not possible (Alderton *et al.*, 2001).

Non-pharmacological countermeasures that have demonstrated some degree of effectiveness - while presumably not having selective effects on cephalic vs. caudal eNOS - act via reversal of the cephalad fluid shift and restoration of the normal hydrostatic pressure gradient exerted on the arterial vasculature along the cephalocaudal axis (Convertino, 2002; Watenpaugh & Hargens, 1996; Watenpaugh *et al.*, 2007). As discussed in the introduction, various methods for restoring the hydrostatic pressure gradient have been demonstrated (including centrifugation, exercise, and use of LBNP), the main limitation currently being the size/weight of apparatus required and the long duration of such countermeasures (Clement & Pavy-Le Traon, 2004; Convertino, 2005; Sun *et al.*, 2002; Watenpaugh *et al.*, 2007; Zhang *et al.*, 2003).

While no direct implications for pharmacological countermeasures arise from the work presented in this thesis, increased knowledge of the physiological effects of simulated or actual microgravity could potentially result in successful future countermeasures. Moreover, the adaptations of individual physiological systems are not thought to occur independently of each other (Nicogossian *et al.*, 1994; Watenpaugh & Hargens, 1996), so increased understanding of the adaptation of one system may facilitate the development of a countermeasure for another. For example, adaptation of cephalic (i.e. above the level of the heart) parts of the skeletal system may occur through changes in cranial pressure and/or other haemodynamic factors (Oganov *et al.*, 1992; Roer & Dillaman, 1990). This implies that pharmacological prevention/treatment of such changes may be beneficial to both cardiovascular and skeletal components. Furthermore, some researchers have proposed that one common countermeasure for all physiological

adaptations to microgravity may be possible, such is the degree of inter-relativity between such adaptations (Convertino, 2005; Zhang, 2001).

4.2.2 Development of novel techniques

In addition to the novel findings relating to differential functional adaptation of the L-arginine/nitric oxide pathway following simulated microgravity, this thesis utilizes several new methods/novel applications of existing techniques for integrative cardiovascular research.

Following the previous *in vitro* observations of differential adaptation in response to HDT (discussed at length in the *Introduction*), corresponding *in vivo* measurements pertaining to the regulation of tone in vascular beds at either end of the cephalocaudal axis were considered of critical importance for this research project. As mentioned previously, the literature relating to *in vivo* investigation of cardiovascular adaptation to microgravity is sparse; reports of simultaneous measurements of blood flow/tissue perfusion in multiple vascular beds following HDT are even rarer. Wilkerson and colleagues utilized radiolabeled microsphere tracers and quantitative autoradiography to demonstrate higher basal vascular resistance and lower blood flow (during head up tilt) in 21 out of 38 discrete regions of the brain in HDT rats compared with controls (Wilkerson *et al.*, 2005). While this technique provides a high degree of spatial resolution, the number of repeated measures in the same animal is limited by the number of differently radiolabeled microspheres that can be measured by quantitative autoradiography (typically 3 or 4), meaning that robust analysis of the dose-response relationships of vasoactive drugs – a powerful tool for investigating changes in physiological function – is not possible.

Real-time, continuous measurement of flow to a vascular bed may be achieved through the use of ultrasonic flowmetry of the conduit vessel supplying the bed of interest. In the case of the hind limb circulation, the common iliac artery may be used for this purpose, and together with simultaneous measurement of systemic arterial pressure has been used as an index of hind

limb vascular resistance/conductance (Miki *et al.*, 2004; Ruble *et al.*, 2002). Measurement of total cerebral vascular resistance using this method is generally not feasible however, as simultaneous measurement of flow through both carotid and vertebral arteries would be necessary. As an alternative, common carotid artery flow, as an index of cerebral blood flow, has been successfully utilized for the measurement of cerebral vascular resistance/conductance (Bailliart *et al.*, 1993; Blanco *et al.*, 1997). Very few studies (if any) have combined ultrasonic measurements of cerebral and hind limb (certainly in the area of cardiovascular adaptation to microgravity) despite the validation of this method for predicting orthostatic intolerance in response to LBNP in humans (Tobal *et al.*, 2001). As demonstrated in the findings of this thesis, measurements of $C_{carotid}$ and C_{iliac} provide suitable indices for cerebral and hind limb vascular tone, and their relatively straightforward use (compared to 'cranial window' techniques for example) makes them a valuable tool for *in vivo* research into differential arterial adaptation following simulated microgravity.

Until fairly recently, the real-time measurement of circulating plasma nitric oxide concentrations has been largely under-utilized in vascular nitric oxide research, primarily due to the extensive technical difficulties associated with earlier nitric oxide sensors, and a lack of commercially available alternatives. During the last five years, at least three different commercial sources of more robust, reliable nitric oxide sensors have been introduced to the scientific community. Despite this, *in vivo* measurements of nitric oxide still remain technically challenging, reflected by the extremely small percentage of the total literature relating to vascular nitric oxide constituted by *in vivo* measurements. Of the handful of laboratories in the world performing such measurements, Mochizuki and colleagues have arguably been the most successful, demonstrating measurement of nitric oxide in rats and dogs, in response to both nitric oxide donors and stimulators of endothelial nitric oxide production (Mochizuki *et al.*,

2002; Mochizuki *et al.*, 2003). Based on such experiments it was considered that application of this technique to the research described in this thesis would provide invaluable data regarding systemic nitric oxide handling following HDT. The high temporal resolution of amperometric nitric oxide measurements compared with classical methods of NO measurement (such as the Griess reaction) allows quantification of the rate of removal of nitric oxide from plasma. Calculation of the decay time constant for the decline in blood nitric oxide concentration (from steady state) upon cessation of a SNP infusion is proposed as an index of the activity of total NO removal pathways. To our knowledge, this technique has been previously reported as part of *in vitro* studies of NO removal pathways (Hakim *et al.*, 1996), but not as part of *in vivo* studies. The information such experiments will provide may obviously contribute significantly to the understanding of how NO metabolism is affected by simulated microgravity, and has further reaching implications for other conditions or diseases where changes in the bioavailability of nitric oxide is thought to contribute to their aetiology (Desjardins & Balligand, 2006; Elmarakby *et al.*, 2003; Price *et al.*, 2000).

The superior sensitivity (~0.1 nM detection limit) of amperometric nitric oxide detection was also exploited in the research discussed in this thesis to measure nitric oxide production from isolated artery perfusate. The custom built apparatus (illustrated in Appendix 2) allowed agonists/antagonists to be perfused at different concentrations in order to construct concentration-response curves. Used together with biochemical measurements of NOS protein expression, this technique represents a powerful method for investigating the nitric oxide producing capacity of isolated arteries in control animals and disease models.

4.2.3 Relevance to orthostatic intolerance unrelated to microgravity

Aside from the direct benefit to the small percentage of the population that are exposed to microgravity environments for prolonged periods of time, increased understanding of how

cardiovascular adaptation may occur will likely provide new opportunities for the development of effective countermeasures. Expanding this knowledge to the wider population, understanding to a greater extent the complexity of the cardiovascular system's response to orthostatic challenge will surely be of great benefit to the many individuals suffering from chronic orthostatic intolerance; the estimated prevalence is over 500,000 in the U.S., and accounts for 3% to 5% of emergency room visits and 1% to 3% of hospital admissions (Robertson *et al.*, 2000; Silverstein *et al.*, 1982). Indeed, there has been a general shift in the understanding of orthostatic intolerance as primarily a result of hypotension. Ultimately, syncope occurs from hypoperfusion of critical areas of the brain, such as the reticular activating system (Franco Folino, 2007). While hypotension reduces the driving force for cerebral perfusion, increasing evidence – including from studies following spaceflight – suggests an emerging role for dysfunction of cerebral autoregulation as a cause of orthostatic intolerance, particularly when pre-syncope/syncope is observed in the absence of marked hypotension, a condition increasingly referred to as *cerebral syncope* (Brignole *et al.*, 2004; Buckey *et al.*, 1996; Franco Folino, 2007). The findings presented in this thesis add further evidence to this hypothesis.

Nonetheless, it should still be appreciated that orthostatic intolerance following spaceflight or HDBR differs from its clinical counterparts by being only transiently present in subjects who otherwise have relatively normal cardiovascular and regulatory systems. However, when applied to the general population, the same set of basic pathophysiological elements should be considered in the analysis of any form of orthostatic intolerance (Blomqvist *et al.*, 1994). Therefore a basic understanding of cardiovascular space research can thus also lead to a better understanding of this clinical situation and possible help patients with chronic orthostatic intolerance.

4.3 SUMMARY & CONCLUSIONS

In summary, the research described in this thesis was performed to address the overall hypothesis that differential adaptation of the vascular L-arginine/nitric oxide pathway occurs in arteries in response to simulated microgravity, such that an increased contribution of endothelium-derived nitric oxide to vascular tone is observed in the peripheral vasculature (caudal to the hydrostatic indifference point), while a decreased contribution of endothelium-derived nitric oxide to vascular tone is observed in the cerebral vasculature (cephalic to the hydrostatic indifference point). The key findings from this research are that following 14 days of simulated microgravity (HDT):

1. Autoregulatory control of carotid blood flow *in vivo* in response to lower body negative pressure is impaired.
2. α_1-Adrenoceptor-mediated vasoconstriction *in vitro* is decreased at the level of the common carotid artery (potentially due to decreased receptor reserve), yet α_1-adrenoceptor-mediated vasoconstriction of the carotid vascular bed is increased *in vivo*. α_1-Adrenoceptor-mediated vasoconstriction is unchanged at the level of the common iliac artery, but is decreased in the iliac vascular bed. Furthermore, β_2-adrenoceptor-mediated vasodilatation *in vivo* remains unaltered.
3. The vascular response to either stimulation of eNOS with acetylcholine or inhibition of eNOS with L-NAME *in vivo* is decreased in the carotid vascular bed but increased in the iliac vascular bed. Inhibition of iNOS with 1400W has no effect on the cardiovascular variables measured.

4. The release of NO in response to stimulation with acetylcholine *in vitro* is decreased in the carotid arteries but remains unchanged in iliac arteries. The decreased response in the carotid arteries is not associated with changes in eNOS protein expression.

5. The *in vivo* vascular response to exogenously administered nitric oxide (via SNP) remains unchanged. The pharmacokinetic half-life of nitric oxide in the circulation and pharmacodynamic response to nitric oxide also remain unchanged.

In conclusion, the above findings suggest that simulated microgravity produces differential changes to the arterial vasculature in according to anatomical region. Using the vascular bed supplied by the common carotid artery to represent the cephalic vasculature and the vascular bed supplied by the common iliac artery to represent the caudal vasculature, it appears that the balance of vasoconstrictor *vs.* vasodilator tone is altered such that in the cephalic vasculature, vascular tone is increased while in the caudal vasculature, tone is decreased. The findings from this research project suggest that changes in the contribution of eNOS to vascular tone in these beds may play a role in these adaptations. In relation to the initial observations regarding the cardiovascular adaptation to simulated or actual microgravity, these findings may go some way to explain why that in human studies of orthostatic intolerance associated with cardiovascular deconditioning (either in response to space flight or prolonged bed rest), pre-syncopal and syncopal symptoms may be observed in the absence of hypotension. Inadequate cerebral perfusion due to the adaptation of the cephalic vasculature in response to the increased transmural pressure experienced in these vessels during simulated/actual microgravity may contribute to this phenomenon.

Orthostatic intolerance following exposure to simulated or actual microgravity likely has several possible mechanisms, including hypovolaemia, carotid-cardiac baroreflex dysfunction,

the inability to raise total peripheral resistance and impaired cerebral autoregulation. The relative contribution of these mechanisms likely varies between individuals, and with different durations of microgravity exposure. This is manifested by the observation that at least three different haemodynamic profiles are associated with presyncope/syncope following spaceflight/HDBR: that associated with sudden hypotension (suggestive of a vasovagal response); following a slow, steady decrease in arterial blood pressure; and that observed in the absence of hypotension (Buckey *et al.*, 1996; Pavy-Le Traon *et al.*, 2007; Watenpaugh & Hargens, 1996). The differential adaptation of the L-arginine/nitric oxide pathway demonstrated by the findings described in this thesis, if the occur in humans, could account for the inability to raise total peripheral resistance and impaired cerebral autoregulation.

REFERENCES

ALDERTON, W.K., COOPER, C.E. & KNOWLES, R.G. (2001). Nitric oxide synthases: structure, function and inhibition. *Biochem J*, **357,** 593-615.

ALFREY, C.P., UDDEN, M.M., LEACH-HUNTOON, C., DRISCOLL, T. & PICKETT, M.H. (1996). Control of red blood cell mass in spaceflight. *J Appl Physiol*, **81,** 98-104.

ARBEILLE, P., ACHAIBOU, F., FOMINA, G., POTTIER, J.M. & PORCHER, M. (1996). Regional blood flow in microgravity: adaptation and deconditioning. *Med Sci Sports Exerc*, **28,** S70-9.

ARBEILLE, P., KERBECI, P., MATTAR, L., SHOEMAKER, J.K. & HUGHSON, R.L. (2008). WISE-2005: tibial and gastrocnemius vein and calf tissue response to LBNP after a 60-day bed rest with and without countermeasures. *J Appl Physiol*, **104,** 938-43.

ASANO, K., CHEE, C.B., GASTON, B., LILLY, C.M., GERARD, C., DRAZEN, J.M. & STAMLER, J.S. (1994). Constitutive and inducible nitric oxide synthase gene expression, regulation, and activity in human lung epithelial cells. *Proc Natl Acad Sci U S A*, **91,** 10089-93.

AUBERT, A.E., BECKERS, F. & VERHEYDEN, B. (2005). Cardiovascular function and basics of physiology in microgravity. *Acta Cardiol*, **60,** 129-51.

BAILLIART, O., BONNIN, P., CAPDEROU, A., SAVIN, E., KEDRA, A.W. & MARTINEAUD, J.P. (1993). Simultaneous ultrasonic measurement of carotid blood flow and intracerebral haemodynamics in man. *Arch Int Physiol Biochim Biophys*, **101,** 149-54.

BAUSER-HEATON, H.D. & BOHLEN, H.G. (2007). Cerebral microvascular dilation during hypotension and decreased oxygen tension: a role for nNOS. *Am J Physiol Heart Circ Physiol*, **293,** H2193-201.

BECKERS, F., SEPS, B., RAMAEKERS, D., VERHEYDEN, B. & AUBERT, A.E. (2003). Parasympathetic heart rate modulation during parabolic flights. *Eur J Appl Physiol*, **90,** 83-91.

BEDFORD, T.G. & TIPTON, C.M. (1985). Lower body negative pressure in the tranquilized rat. *Aviat Space Environ Med*, **56,** 786-90.

BEECH, D.J., ZHANG, H., NAKAO, K. & BOLTON, T.B. (1993). K channel activation by nucleotide diphosphates and its inhibition by glibenclamide in vascular smooth muscle cells. *Br J Pharmacol*, **110,** 573-82.

BEHNKE, B.J., ZAWIEJA, D.C., GASHEV, A.A., RAY, C.A. & DELP, M.D. (2008). Diminished mesenteric vaso- and venoconstriction and elevated plasma ANP and BNP with simulated microgravity. *J Appl Physiol*, **104,** 1273-80.

BELIN DE CHANTEMELE, E., PASCAUD, L., CUSTAUD, M.A., CAPRI, A., LOUISY, F., FERRETTI, G., GHARIB, C. & ARBEILLE, P. (2004). Calf venous volume during stand-test after a 90-day bed-rest study with or without exercise countermeasure. *J Physiol*, **561,** 611-22.

BENDALL, J.K., ALP, N.J., WARRICK, N., CAI, S., ADLAM, D., ROCKETT, K., YOKOYAMA, M., KAWASHIMA, S. & CHANNON, K.M. (2005). Stoichiometric relationships between endothelial tetrahydrobiopterin, endothelial NO synthase (eNOS) activity, and eNOS coupling in vivo: insights from transgenic mice with endothelial-targeted GTP cyclohydrolase 1 and eNOS overexpression. *Circ Res*, **97,** 864-71.

BENHAM, C.D., BOLTON, T.B., LANG, R.J. & TAKEWAKI, T. (1986). Calcium-activated potassium channels in single smooth muscle cells of rabbit jejunum and guinea-pig mesenteric artery. *J Physiol*, **371,** 45-67.

BEVAN, J.A. & OSHER, J.V. (1972). A direct method for recording tension changes in the wall of small blood vessels in vitro. *Agents Actions*, **2,** 257-60.

BIANCHI, A., DE SCHAEPDRYVER, A.F., DE VLEESCHHOUWER, G.R., PREZIOSI, P. & STORMORKEN, H. (1959). On the pharmacology of methoxamine. *Arch Int Pharmacodyn Ther*, **120,** 495-7.

BIKLE, D.D., HALLORAN, B.P. & MOREY-HOLTON, E. (1997). Space flight and the skeleton: lessons for the earthbound. *Endocrinologist*, **7,** 10-22.

BLACKMAN, D.J., MORRIS-THURGOOD, J.A., ATHERTON, J.J., ELLIS, G.R., ANDERSON, R.A., COCKCROFT, J.R. & FRENNEAUX, M.P. (2000). Endothelium-derived nitric oxide contributes to the regulation of venous tone in humans. *Circulation*, **101,** 165-70.

BLANCO, C.E., GIUSSANI, D.A., RIQUELME, R.A., HANSON, M.A. & LLANOS, A.J. (1997). Carotid blood flow changes with behavioral states in the late gestation llama fetus in utero. *Brain Res Dev Brain Res*, **104,** 137-41.

BLATTER, L.A. & WIER, W.G. (1994). Nitric oxide decreases [Ca2+]i in vascular smooth muscle by inhibition of the calcium current. *Cell Calcium*, **15,** 122-31.

BLATZ, A.L. & MAGLEBY, K.L. (1986). Single apamin-blocked Ca-activated K+ channels of small conductance in cultured rat skeletal muscle. *Nature*, **323,** 718-20.

BLOMQVIST, C.G., BUCKEY, J.C., GAFFNEY, F.A., LANE, L.D., LEVINE, B.D. & WATENPAUGH, D.E. (1994). Mechanisms of post-flight orthostatic intolerance. *J Gravit Physiol*, **1,** P122-4.

BLOMQVIST, C.G., STONE, H.L., SHEPHERD, J.T. & ABBOUD, F.M. (1983). In *Cardiovascular adjustments to gravitational stress*. pp. 1025-1063. Bethesda, MD: American Physiological Society.

BOLOTINA, V.M., NAJIBI, S., PALACINO, J.J., PAGANO, P.J. & COHEN, R.A. (1994). Nitric oxide directly activates calcium-dependent potassium channels in vascular smooth muscle. *Nature*, **368,** 850-3.

BONNIN, P., BEN DRISS, A., BENESSIANO, J., MAILLET, A., PAVY LE TRAON, A. & LEVY, B.I. (2001). Enhanced flow-dependent vasodilatation after bed rest, a possible mechanism for orthostatic intolerance in humans. *Eur J Appl Physiol*, **85,** 420-6.

BOULANGER, C. & LUSCHER, T.F. (1990). Release of endothelin from the porcine aorta. Inhibition by endothelium-derived nitric oxide. *J Clin Invest*, **85,** 587-90.

BOULANGER, C.M., MORRISON, K.J. & VANHOUTTE, P.M. (1994). Mediation by M3-muscarinic receptors of both endothelium-dependent contraction and relaxation to acetylcholine in the aorta of the spontaneously hypertensive rat. *Br J Pharmacol*, **112,** 519-24.

BRAYDEN, J.E. & NELSON, M.T. (1992). Regulation of arterial tone by activation of calcium-dependent potassium channels. *Science*, **256,** 532-5.

BREDT, D.S. & SNYDER, S.H. (1990). Isolation of nitric oxide synthetase, a calmodulin-requiring enzyme. *Proc Natl Acad Sci U S A*, **87,** 682-5.

BRENMAN, J.E., CHAO, D.S., GEE, S.H., MCGEE, A.W., CRAVEN, S.E., SANTILLANO, D.R., WU, Z., HUANG, F., XIA, H., PETERS, M.F., FROEHNER, S.C. & BREDT, D.S. (1996). Interaction of nitric oxide synthase with the postsynaptic density protein PSD-95 and alpha1-syntrophin mediated by PDZ domains. *Cell*, **84,** 757-67.

BRIGNOLE, M., ALBONI, P., BENDITT, D.G., BERGFELDT, L., BLANC, J.J., BLOCH THOMSEN, P.E., VAN DIJK, J.G., FITZPATRICK, A., HOHNLOSER, S., JANOUSEK, J., KAPOOR, W., KENNY, R.A., KULAKOWSKI, P., MASOTTI, G., MOYA, A., RAVIELE, A., SUTTON, R., THEODORAKIS, G., UNGAR, A. & WIELING, W. (2004). Guidelines on management (diagnosis and treatment) of syncope--update 2004. *Europace*, **6,** 467-537.

BUCCI, M., GRATTON, J.P., RUDIC, R.D., ACEVEDO, L., ROVIEZZO, F., CIRINO, G. & SESSA, W.C. (2000). In vivo delivery of the caveolin-1 scaffolding domain inhibits nitric oxide synthesis and reduces inflammation. *Nat Med*, **6,** 1362-7.

BUCKEY, J.C., GAFFNEY, F.A., LANE, L.D., LEVINE, B.D., WATENPAUGH, D.E. & BLOMQVIST, C.G. (1993). Central venous pressure in space. *N Engl J Med*, **328,** 1853-4.

BUCKEY, J.C., JR., LANE, L.D., LEVINE, B.D., WATENPAUGH, D.E., WRIGHT, S.J., MOORE, W.E., GAFFNEY, F.A. & BLOMQVIST, C.G. (1996). Orthostatic intolerance after spaceflight. *J Appl Physiol*, **81,** 7-18.

BUCKEY, J.C., PESHOCK, R.M. & BLOMQVIST, C.G. (1988). Deep venous contribution to hydrostatic blood volume change in the human leg. *Am J Cardiol*, **62,** 449-53.

BUNGO, M.W., CHARLES, J.B. & JOHNSON, P.C., JR. (1985). Cardiovascular deconditioning during space flight and the use of saline as a countermeasure to orthostatic intolerance. *Aviat Space Environ Med*, **56,** 985-90.

BUNGO, M.W. & JOHNSON, P.C., JR. (1983). Cardiovascular examinations and observations of deconditioning during the space shuttle orbital flight test program. *Aviat Space Environ Med*, **54,** 1001-4.

BUSCONI, L. & MICHEL, T. (1993). Endothelial nitric oxide synthase. N-terminal myristoylation determines subcellular localization. *J Biol Chem*, **268,** 8410-3.

BUSSE, R., FLEMING, I. & MAYER, B. (2000). In *Nitric oxide and the regulation of vascular tone*. pp. 179-206. Berlin ; New York: Springer.

CAILLOT-AUGUSSEAU, A., LAFAGE-PROUST, M.H., SOLER, C., PERNOD, J., DUBOIS, F. & ALEXANDRE, C. (1998). Bone formation and resorption biological markers in cosmonauts during and after a 180-day space flight (Euromir 95). *Clin Chem*, **44,** 578-85.

CALVIN, M. & GAZENKO, O.G. (1975). Foundations of Space Biology & Medicine. *Scientific and Technical Office, National Aeronautics and Space Administration. Washington, DC*.

CAMERON, N.E. & COTTER, M.A. (1996). Rapid reversal by aminoguanidine of the neurovascular effects of diabetes in rats: modulation by nitric oxide synthase inhibition. *Metabolism*, **45,** 1147-52.

CARLSSON, S.I., BERTILACCIO, M.T., ASCARI, I., BRADAMANTE, S. & MAIER, J.A. (2002). Modulation of human endothelial cell behaviour in simulated microgravity. *J Gravit Physiol*, **9,** P273-4.

CHARLES, J.B., BUNGO, M.W., FORTNER, G.W., NICOGOSSIAN, A.E., HUNTOON, C.L. & POOL, S.L. (1994). In *Physiologic adaptation to spaceflight: Cardiopulmonary Function*. pp. 286-304. Philadelphia: Lea & Fibiger.

CHARLES, J.B. & LATHERS, C.M. (1991). Cardiovascular adaptation to spaceflight. *J Clin Pharmacol*, **31,** 1010-23.

CHEW, H.G., JR. & SEGAL, S.S. (1997). Arterial morphology and blood volumes of rats following 10-14 weeks of tail suspension. *Med Sci Sports Exerc*, **29,** 1304-10.

CHRISTENSEN, N.J., DRUMMER, C. & NORSK, P. (2001). Renal and sympathoadrenal responses in space. *Am J Kidney Dis*, **38,** 679-83.

CLEMENT, G. & PAVY-LE TRAON, A. (2004). Centrifugation as a countermeasure during actual and simulated microgravity: a review. *Eur J Appl Physiol*, **92,** 235-48.

COHEN, A.W., HNASKO, R., SCHUBERT, W. & LISANTI, M.P. (2004). Role of caveolae and caveolins in health and disease. *Physiol Rev*, **84,** 1341-79.

COLLERAN, P.N., BEHNKE, B.J., WILKERSON, M.K., DONATO, A.J. & DELP, M.D. (2008). Simulated microgravity alters rat mesenteric artery vasoconstrictor dynamics through an intracellular Ca(2+) release mechanism. *Am J Physiol Regul Integr Comp Physiol*, **294,** R1577-85.

COLLERAN, P.N., WILKERSON, M.K., BLOOMFIELD, S.A., SUVA, L.J., TURNER, R.T. & DELP, M.D. (2000). Alterations in skeletal perfusion with simulated microgravity: a possible mechanism for bone remodeling. *J Appl Physiol*, **89,** 1046-54.

CONVERTINO, V.A. (2005). Consequences of cardiovascular adaptation to spaceflight: implications for the use of pharmacological countermeasures. *Gravit Space Biol Bull*, **18,** 59-69.

CONVERTINO, V.A. (1998). High sustained +Gz acceleration: physiological adaptation to high-G tolerance. *J Gravit Physiol*, **5,** P51-4.

CONVERTINO, V.A. (2001). Lower body negative pressure as a tool for research in aerospace physiology and military medicine. *J Gravit Physiol*, **8,** 1-14.

CONVERTINO, V.A. (2002). Mechanisms of microgravity induced orthostatic intolerance: implications for effective countermeasures. *J Gravit Physiol*, **9,** 1-13.

CONVERTINO, V.A., DOERR, D.F., ECKBERG, D.L., FRITSCH, J.M. & VERNIKOS-DANELLIS, J. (1990). Head-down bed rest impairs vagal baroreflex responses and provokes orthostatic hypotension. *J Appl Physiol*, **68,** 1458-64.

CONVERTINO, V.A., DOERR, D.F., FLORES, J.F., HOFFLER, G.W. & BUCHANAN, P. (1988). Leg size and muscle functions associated with leg compliance. *J Appl Physiol*, **64,** 1017-21.

CONVERTINO, V.A., DOERR, D.F., LUDWIG, D.A. & VERNIKOS, J. (1994). Effect of simulated microgravity on cardiopulmonary baroreflex control of forearm vascular resistance. *Am J Physiol*, **266,** R1962-9.

CONVERTINO, V.A., POLET, J.L., ENGELKE, K.A., HOFFLER, G.W., LANE, L.D. & BLOMQVIST, C.G. (1997). Evidence for increased beta-adrenoreceptor responsiveness induced by 14 days of simulated microgravity in humans. *Am J Physiol*, **273,** R93-9.

COOKE, W.H., AMES, J.E.I., CROSSMAN, A.A., COX, J.F., KUUSELA, T.A., TAHVANAINEN, K.U., MOON, L.B., DRESCHER, J., BAISCH, F.J., MANO, T., LEVINE, B.D., BLOMQVIST, C.G. & ECKBERG, D.L. (2000). Nine months in space: effects on human autonomic cardiovascular regulation. *J Appl Physiol*, **89,** 1039-45.

CRANDALL, C.G., ENGELKE, K.A., CONVERTINO, V.A. & RAVEN, P.B. (1994). Aortic baroreflex control of heart rate after 15 days of simulated microgravity exposure. *J Appl Physiol*, **77,** 2134-9.

DAVIDGE, S.T., BAKER, P.N., LAUGHLIN, M.K. & ROBERTS, J.M. (1995). Nitric oxide produced by endothelial cells increases production of eicosanoids through activation of prostaglandin H synthase. *Circ Res*, **77,** 274-83.

DAVIS, J.R., VANDERPLOEG, J.M., SANTY, P.A., JENNINGS, R.T. & STEWART, D.F. (1988). Space motion sickness during 24 flights of the space shuttle. *Aviat Space Environ Med*, **59,** 1185-9.

DE SALVATORE, G., DESAPHY, J.F., PIEPOLI, A.L., NATALE, L., DE SALVIA, M.A., MITOLO, C.I., RENNA, G., CONTE-CAMERINO, D. & MITOLO-CHIEPPA, D. (2004). Functional alterations of mesenteric vascular bed, vas deferens and intestinal tracts in a rat hindlimb unloading model of microgravity. *Auton Autacoid Pharmacol*, **24,** 45-54.

DELP, M.D. (1999). Myogenic and vasoconstrictor responsiveness of skeletal muscle arterioles is diminished by hindlimb unloading. *J Appl Physiol*, **86,** 1178-84.

DELP, M.D., BROWN, M., LAUGHLIN, M.H. & HASSER, E.M. (1995). Rat aortic vasoreactivity is altered by old age and hindlimb unloading. *J Appl Physiol*, **78,** 2079-86.

DELP, M.D., COLLERAN, P.N., WILKERSON, M.K., MCCURDY, M.R. & MULLER-DELP, J. (2000). Structural and functional remodeling of skeletal muscle microvasculature is induced by simulated microgravity. *Am J Physiol Heart Circ Physiol*, **278,** H1866-73.

DELP, M.D., HOLDER-BINKLEY, T., LAUGHLIN, M.H. & HASSER, E.M. (1993). Vasoconstrictor properties of rat aorta are diminished by hindlimb unweighting. *J Appl Physiol*, **75,** 2620-8.

DEMIOT, C., DIGNAT-GEORGE, F., FORTRAT, J.O., SABATIER, F., GHARIB, C., LARINA, I., GAUQUELIN-KOCH, G., HUGHSON, R. & CUSTAUD, M.A. (2007). WISE 2005: chronic bed rest impairs microcirculatory endothelium in women. *Am J Physiol Heart Circ Physiol*, **293,** H3159-64.

DENNINGER, J.W. & MARLETTA, M.A. (1999). Guanylate cyclase and the .NO/cGMP signaling pathway. *Biochim Biophys Acta*, **1411,** 334-50.

DESJARDINS, F. & BALLIGAND, J.L. (2006). Nitric oxide-dependent endothelial function and cardiovascular disease. *Acta Clin Belg*, **61,** 326-34.

DI PRAMPERO, P.E. & ANTONUTTO, G. (1997). Cycling in space to simulate gravity. *Int J Sports Med*, **18 Suppl 4,** S324-6.

DIETRICH, J.E., WHEDON, G.D., SHORR, E., TOSCANI, V. & DAVIS, V.B. (1948). Effect of immobilisation on metabolic and physiologic functions of normal men. *Am J Med*, **4,** 3-35.

DINERMAN, J.L., DAWSON, T.M., SCHELL, M.J., SNOWMAN, A. & SNYDER, S.H. (1994). Endothelial nitric oxide synthase localized to hippocampal pyramidal cells: implications for synaptic plasticity. *Proc Natl Acad Sci U S A*, **91,** 4214-8.

DRUMMER, C., HEER, M., DRESSENDORFER, R.A., STRASBURGER, C.J. & GERZER, R. (1993). Reduced natriuresis during weightlessness. *Clin Investig*, **71,** 678-86.

DRUMMER, C., HESSE, C., BAISCH, F., NORSK, P., ELMANN-LARSEN, B., GERZER, R. & HEER, M. (2000). Water and sodium balances and their relation to body mass changes in microgravity. *Eur J Clin Invest*, **30,** 1066-75.

DRUMMER, C., NORSK, P. & HEER, M. (2001). Water and sodium balance in space. *Am J Kidney Dis*, **38,** 684-90.

DUNBAR, S.L., BERKOWITZ, D.E., BROOKS-ASPLUND, E.M. & SHOUKAS, A.A. (2000). The effects of hindlimb unweighting on the capacitance of rat small mesenteric veins. *J Appl Physiol*, **89,** 2073-7.

DUNBAR, S.L., TAMHIDI, L., BERKOWITZ, D.E. & SHOUKAS, A.A. (2001). Hindlimb unweighting affects rat vascular capacitance function. *Am J Physiol Heart Circ Physiol*, **281,** H1170-7.

DUNN, C.D., JOHNSON, P.C., LANGE, R.D., PEREZ, L. & NESSEL, R. (1985). Regulation of hematopoiesis in rats exposed to antiorthostatic, hypokinetic/hypodynamia: I. Model description. *Aviat Space Environ Med*, **56,** 419-26.

EATMAN, D., LISTHROP, R.A., BEASLEY, A.S., SOCCI, R.R., ABUKHALAF, I. & BAYORH, M.A. (2003a). Influences of prostanoids and nitric oxide on post-suspension hypotension in female Sprague-Dawley rats. *Prostaglandins Leukot Essent Fatty Acids*, **68,** 197-205.

EATMAN, D., WALTON, M., SOCCI, R.R., EMMETT, N. & BAYORH, M.A. (2003b). NOS II inhibition attenuates post-suspension hypotension in Sprague-Dawley rats. *Clin Exp Hypertens*, **25,** 11-24.

ECKBERG, D.L. (2003). Bursting into space: alterations of sympathetic control by space travel. *Acta Physiol Scand*, **177,** 299-311.

EDGERTON, V.R. & ROY, R.R. (2000). Invited review: gravitational biology of the neuromotor systems: a perspective to the next era. *J Appl Physiol*, **89,** 1224-31.

ELLIOTT, A.R., PRISK, G.K., GUY, H.J., KOSONEN, J.M. & WEST, J.B. (1996). Forced expirations and maximum expiratory flow-volume curves during sustained microgravity on SLS-1. *J Appl Physiol*, **81,** 33-43.

ELMARAKBY, A.A., WILLIAMS, J.M. & POLLOCK, D.M. (2003). Targeting sources of superoxide and increasing nitric oxide bioavailability in hypertension. *Curr Opin Investig Drugs*, **4,** 282-90.

ENGELKE, K.A., DOERR, D.F. & CONVERTINO, V.A. (1995). A single bout of exhaustive exercise affects integrated baroreflex function after 16 days of head-down tilt. *Am J Physiol*, **269,** R614-20.

FADIUKOVA, O.E., TARASOVA, O.S. & VINOGRADOVA, O.L. (2005). [Effect of two-week tail suspension on the reactivity of rat's cerebral vessels]. *Aviakosm Ekolog Med*, **39,** 23-7.

FARACI, F.M. (1990). Role of nitric oxide in regulation of basilar artery tone in vivo. *Am J Physiol*, **259,** H1216-21.

FARACI, F.M., BRIAN, J.E., JR. & HEISTAD, D.D. (1995). Response of cerebral blood vessels to an endogenous inhibitor of nitric oxide synthase. *Am J Physiol*, **269,** H1522-7.

FELETOU, M. & VANHOUTTE, P.M. (1996). Endothelium-derived hyperpolarizing factor. *Clin Exp Pharmacol Physiol*, **23,** 1082-90.

FERGUSON, J.K., TAYLOR, G.R., MIESZKUC, B.J., JOHNSON, R.S., DIETLEIN, L.F. & BERRY, C.A. (1975). In *Microbial investigations*. pp. 83-103. Washington, DC: U.S. Government printing office.

FITTS, R.H., RILEY, D.R. & WIDRICK, J.J. (2001). Functional and structural adaptations of skeletal muscle to microgravity. *J Exp Biol*, **204,** 3201-8.

FITTS, R.H., RILEY, D.R. & WIDRICK, J.J. (2000). Physiology of a microgravity environment invited review: microgravity and skeletal muscle. *J Appl Physiol*, **89,** 823-39.

FÖRSTERMANN, U. & MAYER, B. (2000). In *Regulation of Nitric Oxide Synthase Expression and Activity*. pp. 71-91. Berlin ; New York: Springer.

FÖRSTERMANN, U., POLLOCK, J.S., SCHMIDT, H.H., HELLER, M. & MURAD, F. (1991). Calmodulin-dependent endothelium-derived relaxing factor/nitric oxide synthase activity is present in the particulate and cytosolic fractions of bovine aortic endothelial cells. *Proc Natl Acad Sci U S A*, **88,** 1788-92.

FRANCO FOLINO, A. (2007). Cerebral autoregulation and syncope. *Prog Cardiovasc Dis*, **50,** 49-80.

FREY, M.A. (1996). Space research activities during missions of the past. *Med Sci Sports Exerc*, **28,** S3-8.

FRITSCH-YELLE, J.M., CHARLES, J.B., JONES, M.M., BEIGHTOL, L.A. & ECKBERG, D.L. (1994). Spaceflight alters autonomic regulation of arterial pressure in humans. *J Appl Physiol*, **77,** 1776-83.

FRITSCH-YELLE, J.M., CHARLES, J.B., JONES, M.M. & WOOD, M.L. (1996a). Microgravity decreases heart rate and arterial pressure in humans. *J Appl Physiol*, **80,** 910-4.

FRITSCH-YELLE, J.M., WHITSON, P.A., BONDAR, R.L. & BROWN, T.E. (1996b). Subnormal norepinephrine release relates to presyncope in astronauts after spaceflight. *J Appl Physiol*, **81,** 2134-41.

FU, Q., WITKOWSKI, S. & LEVINE, B.D. (2004a). Vasoconstrictor reserve and sympathetic neural control of orthostasis. *Circulation*, **110,** 2931-7.

FU, Z.J., XIE, M.J., ZHANG, L.F., CHENG, H.W. & MA, J. (2004b). Differential activation of potassium channels in cerebral and hindquarter arteries of rats during simulated microgravity. *Am J Physiol Heart Circ Physiol*, **287,** H1505-15.

FURCHGOTT, R.F. (1966). The use of beta-haloalkylamines in the differentiation of receptors and the determination of dissociation constants of receptor agonist complexes. *Adv Drug Res*, **3,** 21-55.

GABRIELSEN, A., NORSK, P., VIDEBAEK, R. & HENRIKSEN, O. (1995). Effect of microgravity on forearm subcutaneous vascular resistance in humans. *J Appl Physiol*, **79,** 434-8.

GARCIA-CARDENA, G., OH, P., LIU, J., SCHNITZER, J.E. & SESSA, W.C. (1996). Targeting of nitric oxide synthase to endothelial cell caveolae via palmitoylation: implications for nitric oxide signaling. *Proc Natl Acad Sci U S A*, **93,** 6448-53.

GARVEY, E.P., OPLINGER, J.A., FURFINE, E.S., KIFF, R.J., LASZLO, F., WHITTLE, B.J. & KNOWLES, R.G. (1997). 1400W is a slow, tight binding, and highly selective inhibitor of inducible nitric-oxide synthase in vitro and in vivo. *J Biol Chem*, **272,** 4959-63.

GAUER, O.H. & THRON, H.L. (1965). Postural changes in the circulation. Handbook of Physiology: Circulation. *New York, NY Oxford University Press***,** 2409-2439.

GAZENKO, O.G., GENIN, A.M. & EGOROV, A.D. (1981). Summary of medical investigations in the U.S.S.R. manned space missions. *Acta Astronaut*, **8,** 907-17.

GEARY, G.G., KRAUSE, D.N., PURDY, R.E. & DUCKLES, S.P. (1998). Simulated microgravity increases myogenic tone in rat cerebral arteries. *J Appl Physiol*, **85,** 1615-21.

GIANGIACOMO, K.M., GARCIA, M.L. & MCMANUS, O.B. (1992). Mechanism of iberiotoxin block of the large-conductance calcium-activated potassium channel from bovine aortic smooth muscle. *Biochemistry*, **31,** 6719-27.

GIRISH, V. & VIJAYALAKSHMI, A. (2004). Affordable image analysis using NIH Image/ImageJ. *Indian J Cancer*, **41,** 47.

GLICK, M.R., GEHMAN, J.D. & GASCHO, J.A. (1993). Endothelium-derived nitric oxide reduces baseline venous tone in awake instrumented rats. *Am J Physiol*, **265,** H47-51.

GOLDING, E.M., STEENBERG, M.L., CHERIAN, L., MARRELLI, S.P., ROBERTSON, C.S. & BRYAN, R.M., JR. (1998). Endothelial-mediated dilations following severe controlled cortical impact injury in the rat middle cerebral artery. *J Neurotrauma*, **15,** 635-44.

GRAEBE, A., SCHUCK, E.L., LENSING, P., PUTCHA, L. & DERENDORF, H. (2004). Physiological, pharmacokinetic, and pharmacodynamic changes in space. *J Clin Pharmacol*, **44,** 837-53.

GRAY, S.J. & STERLING, K. (1950). Determination of circulating red cell volume by radioactive chromium. *Science*, **112,** 179-80.

GREENLEAF, J.E., WADE, C.E. & LEFTHERIOTIS, G. (1989). Orthostatic responses following 30-day bed rest deconditioning with isotonic and isokinetic exercise training. *Aviat Space Environ Med*, **60,** 537-42.

GREENWAY, C.V. (1981). Mechanisms and quantitative assessment of drug effects on cardiac output with a new model of the circulation. *Pharmacol Rev*, **33,** 213-51.

GRIGORIEV, A.I., BUGROV, S.A., BOGOMOLOV, V.V., EGOROV, A.D., KOZLOVSKAYA, I.B., PESTOV, I.D., POLYAKOV, V.V. & TARASOV, I.K. (1991). Preliminary medical results of the Mir year-long mission. *Acta Astronaut*, **23,** 1-8.

GRIGORIEV, A.I. & EGOROV, A.D. (1996). The mechanisms involved in cardiovascular system changes during long space flights. *Environ Med*, **40,** 1-11.

GUNDEL, A., DRESCHER, J., SPATENKO, Y.A. & POLYAKOV, V.V. (2002). Changes in basal heart rate in spaceflights up to 438 days. *Aviat Space Environ Med*, **73,** 17-21.

GUYTON, A.C., RICHARDSON, T.Q. & LANGSTON, J.B. (1964). Regulation of cardiac output and venous return. *Clin Anesth*, **3,** 1-34.

HABER, F. & HABER, H. (1950). Possible methods of producing the gravity free state for medical research. *J Aviat Med (Aerosp Med)*, **21,** 395-400.

HAKIM, T.S., SUGIMORI, K., CAMPORESI, E.M. & ANDERSON, G. (1996). Half-life of nitric oxide in aqueous solutions with and without haemoglobin. *Physiol Meas*, **17,** 267-77.

HARA, Y., KITAMURA, K. & KURIYAMA, H. (1980). Actions of 4-aminopyridine on vascular smooth muscle tissues of the guinea-pig. *Br J Pharmacol*, **68,** 99-106.

HARGENS, A.R., STESKAL, J., JOHANSSON, C. & TIPTON, C.M. (1984). Tissue fluid shift, forelimb loading, and tail tension in tail-suspended rats. *Physiologist*, **27,** S37-S38.

HARGENS, A.R., WATENPAUGH, D.E. & BREIT, G.A. (1992). Control of circulatory function in altered gravitational fields. *Physiologist*, **35,** S80-3.

HARGENS, A.R., WHALEN, R.T., WATENPAUGH, D.E., SCHWANDT, D.F. & KROCK, L.P. (1991). Lower body negative pressure to provide load bearing in space. *Aviat Space Environ Med*, **62,** 934-7.

HATTON, D.C., YUE, Q., CHAPMAN, J., XUE, H., DIERICKX, J., ROULLET, C., COSTE, S., ROULLET, J.B. & MCCARRON, D.A. (2002). Blood pressure and mesenteric resistance arterial function after spaceflight. *J Appl Physiol*, **92,** 13-7.

HAWKINS, W.R., ZIEGLESCHMID, J.F., JOHNSON, R.S., DIETLEIN, L.F. & BERRY, C.A. (1975). In *Clinical aspects of crew health*. pp. 43-81. Washington, DC: U.S. Government printing office.

HEISTAD, D.D., KONTOS, H.A., SHEPHERD, J.T. & ABBOUD, F.M. (1983). In *Cerebral circulation*. pp. 137-182. Bethesda, MD: American Physiological Society.

HENRY, J.P., GAUER, O.H. & REEVES, J.L. (1956). Evidence of the atrial location of receptors influencing urine flow. *Circ Res*, **4,** 85-90.

HERAULT, S., FOMINA, G., ALFEROVA, I., KOTOVSKAYA, A., POLIAKOV, V. & ARBEILLE, P. (2000). Cardiac, arterial and venous adaptation to weightlessness during 6-month MIR spaceflights with and without thigh cuffs (bracelets). *Eur J Appl Physiol*, **81,** 384-90.

HESSE, C., SIEDLER, H., LUNTZ, S.P., ARENDT, B.M., GOERLICH, R., FRICKER, R., HEER, M. & HAEFELI, W.E. (2005). Modulation of endothelial and smooth muscle function by bed rest and hypoenergetic, low-fat nutrition. *J Appl Physiol*, **99,** 2196-203.

HEVEL, J.M., WHITE, K.A. & MARLETTA, M.A. (1991). Purification of the inducible murine macrophage nitric oxide synthase. Identification as a flavoprotein. *J Biol Chem*, **266,** 22789-91.

HOFFLER, G.W., JOHNSON, R.L., JOHNSON, R.S., DIETLEIN, L.F. & BERRY, C.A. (1975). In *Apollo flight crew cardiovascular evaluations*. pp. 227-264. Washington, DC: U.S. Government printing office.

HU, E., DUL, E., SUNG, C.M., CHEN, Z., KIRKPATRICK, R., ZHANG, G.F., JOHANSON, K., LIU, R., LAGO, A., HOFMANN, G., MACARRON, R., DE LOS FRAILES, M., PEREZ, P., KRAWIEC, J., WINKLER, J. & JAYE, M. (2003). Identification of novel isoform-selective inhibitors within class I histone deacetylases. *J Pharmacol Exp Ther*, **307,** 720-8.

HUNTOON, C., CINTRÛN, N.M., WHITSON, P.A., NICOGOSSIAN, A.E., HUNTOON, C.L. & POOL, S.L. (1994). In *Physiologic adaptation to spaceflight: Endocrine and Biochemical Functions*. pp. 334-350. Philadelphia: Lea & Fibiger.

HWANG, S., SHELKOVNIKOV, S.A. & PURDY, R.E. (2007). Simulated microgravity effects on the rat carotid and femoral arteries: role of contractile protein expression and mechanical properties of the vessel wall. *J Appl Physiol*, **102,** 1595-603.

IGNARRO, L.J., BUGA, G.M., WOOD, K.S., BYRNS, R.E. & CHAUDHURI, G. (1987). Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide. *Proc Natl Acad Sci U S A*, **84,** 9265-9.

JACKSON, W.F. (1993). Arteriolar tone is determined by activity of ATP-sensitive potassium channels. *Am J Physiol*, **265,** H1797-803.

JASPERSE, J.L., WOODMAN, C.R., PRICE, E.M., HASSER, E.M. & LAUGHLIN, M.H. (1999). Hindlimb unweighting decreases ecNOS gene expression and endothelium-dependent dilation in rat soleus feed arteries. *J Appl Physiol*, **87,** 1476-82.

JAWEED, M.M., NICOGOSSIAN, A.E., HUNTOON, C.L. & POOL, S.L. (1994). In *Physiologic adaptation to spaceflight: Muscle structure and function*. pp. 317-326. Philadelphia: Lea & Fibiger.

JE, H.D., GALLANT, C., LEAVIS, P.C. & MORGAN, K.G. (2004). Caveolin-1 regulates contractility in differentiated vascular smooth muscle. *Am J Physiol Heart Circ Physiol*, **286,** H91-8.

JOHNSON, P.C. (1979). Fluid volumes changes induced by spaceflight. *Acta Astronaut*, **6,** 1335-41.

JOHNSTON, R.S. & DIETLEIN, L.F. (1977). Biomedical results from Skylab. *Scientific and Technical Information Office, National Aeronautics and Space Administration. Washington, DC*.

JONES, S.C., EASLEY, K.A., RADINSKY, C.R., CHYATTE, D., FURLAN, A.J. & PEREZ-TREPICHIO, A.D. (2003). Nitric oxide synthase inhibition depresses the height of the cerebral blood flow-pressure autoregulation curve during moderate hypotension. *J Cereb Blood Flow Metab*, **23,** 1085-95.

KADOWITZ, P.J. & MCNAMARA, D.B. (2000). Nitric oxide and the regulation of the peripheral circulation. *Boston. Birkhauser*.

KATSUKI, S., ARNOLD, W., MITTAL, C. & MURAD, F. (1977). Stimulation of guanylate cyclase by sodium nitroprusside, nitroglycerin and nitric oxide in various tissue preparations and comparison to the effects of sodium azide and hydroxylamine. *J Cyclic Nucleotide Res*, **3,** 23-35.

KAWAI, Y., MURTHY, G., WATENPAUGH, D.E., BREIT, G.A., DEROSHIA, C.W. & HARGENS, A.R. (1993). Cerebral blood flow velocity in humans exposed to 24 h of head-down tilt. *J Appl Physiol*, **74,** 3046-51.

KELMAN, G.R., PALMER, K.N. & CROSS, M.R. (1969). Cardiovascular effects of AH.3365 (salbutamol). *Nature*, **221,** 1251.

KIHARA, M., SCHMELZER, J.D., PODUSLO, J.F., CURRAN, G.L., NICKANDER, K.K. & LOW, P.A. (1991). Aminoguanidine effects on nerve blood flow, vascular permeability, electrophysiology, and oxygen free radicals. *Proc Natl Acad Sci U S A*, **88,** 6107-11.

KIMOTO, M., TSUJI, H., OGAWA, T. & SASAOKA, K. (1993). Detection of NG,NG-dimethylarginine dimethylaminohydrolase in the nitric oxide-generating systems of rats using monoclonal antibody. *Arch Biochem Biophys*, **300,** 657-62.

KIRSCH, K.A., BAARTZ, F.J., GUNGA, H.C. & ROCKER, L. (1993). Fluid shifts into and out of superficial tissues under microgravity and terrestrial conditions. *Clin Investig*, **71,** 687-9.

KIRSCH, K.A., ROCKER, L., GAUER, O.H., KRAUSE, R., LEACH, C., WICKE, H.J. & LANDRY, R. (1984). Venous pressure in man during weightlessness. *Science*, **225,** 218-9.

KNOT, H.J. & NELSON, M.T. (1995). Regulation of membrane potential and diameter by voltage-dependent K+ channels in rabbit myogenic cerebral arteries. *Am J Physiol*, **269,** H348-55.

KOENIG, S.C., CONVERTINO, V.A., FANTON, J.W., REISTER, C.A., GAFFNEY, F.A., LUDWIG, D.A., KROTOV, V.P., TRAMBOVETSKY, E.V. & LATHAM, R.D. (1998). Evidence for increased cardiac compliance during exposure to simulated microgravity. *Am J Physiol*, **275,** R1343-52.

KOMAS, N., LUGNIER, C. & STOCLET, J.C. (1991). Endothelium-dependent and independent relaxation of the rat aorta by cyclic nucleotide phosphodiesterase inhibitors. *Br J Pharmacol*, **104,** 495-503.

KOTANI, K., UENO, S., SANO, A. & KAKIMOTO, Y. (1992). Isolation and identification of methylarginines from bovine brain. *J Neurochem*, **58,** 1127-9.

LASZLO, F., EVANS, S.M. & WHITTLE, B.J. (1995). Aminoguanidine inhibits both constitutive and inducible nitric oxide synthase isoforms in rat intestinal microvasculature in vivo. *Eur J Pharmacol*, **272,** 169-75.

LAUTT, W.W. (1989). Resistance or conductance for expression of arterial vascular tone. *Microvasc Res*, **37,** 230-6.

LAUTT, W.W. (1999). Should clinical cardiologists report total peripheral resistance or total peripheral conductance? *Can J Cardiol*, **15,** 45-7.

LEACH, C.S. (1986). Changes in renal function and fluid and electrolyte regulation in spaceflight. *Book of Abstracts, World Space Congress, Washington DC*, 81.

LEACH, C.S., ALFREY, C.P., SUKI, W.N., LEONARD, J.I., RAMBAUT, P.C., INNERS, L.D., SMITH, S.M., LANE, H.W. & KRAUHS, J.M. (1996). Regulation of body fluid compartments during short-term spaceflight. *J Appl Physiol*, **81,** 105-16.

LEACH, C.S., JOHNSON, P.C. & RAMBAUT, P.C. (1976). Metabolic and endocrine studies: the second manned Skylab mission. *Aviat Space Environ Med*, **47,** 402-10.

LEFFLER, C.W., KADOWITZ, P.J. & MCNAMARA, D.B. (2000). In *Nitric Oxide in Control of the Cerebral Circulation*. pp. 113-127. Boston: Birkhauser.

LEONARD, J.I., LEACH, C.S. & RAMBAUT, P.C. (1983). Quantitation of tissue loss during prolonged space flight. *Am J Clin Nutr*, **38,** 667-79.

LI, G. & CHEUNG, D.W. (1999). Effects of paxilline on K+ channels in rat mesenteric arterial cells. *Eur J Pharmacol*, **372,** 103-7.

LIU, J., GARCIA-CARDENA, G. & SESSA, W.C. (1995). Biosynthesis and palmitoylation of endothelial nitric oxide synthase: mutagenesis of palmitoylation sites, cysteines-15 and/or -26, argues against depalmitoylation-induced translocation of the enzyme. *Biochemistry*, **34,** 12333-40.

LOOFT-WILSON, R.C. & GISOLFI, C.V. (2000). Rat small mesenteric artery function after hindlimb suspension. *J Appl Physiol*, **88,** 1199-206.

LORENZ, M., HEWING, B., HUI, J., ZEPP, A., BAUMANN, G., BINDEREIF, A., STANGL, V. & STANGL, K. (2007). Alternative splicing in intron 13 of the human eNOS gene: a potential mechanism for regulating eNOS activity. *FASEB J*, **21,** 1556-64.

MA, J., KAHWAJI, C.I., NI, Z., VAZIRI, N.D. & PURDY, R.E. (2003). Effects of simulated microgravity on arterial nitric oxide synthase and nitrate and nitrite content. *J Appl Physiol*, **94,** 83-92.

MA, J., ZHANG, L. & YANG, T. (1998a). Alteration of vasoreactivity of mesenteric arteries in rats after two-week simulated weightlessness. *Space Med Med Eng (Beijing)*, **11,** 79-82.

MA, J., ZHANG, L.N., ZHANG, L.F. & YANG, T.D. (1998b). Difference in alterations of adrenoceptor-mediated vasoreactivity of different arteries from tail-suspended rats. *Chin J Aerospace Med*, **9,** 201-204.

MACALLISTER, R.J., FICKLING, S.A., WHITLEY, G.S. & VALLANCE, P. (1994). Metabolism of methylarginines by human vasculature; implications for the regulation of nitric oxide synthesis. *Br J Pharmacol*, **112,** 43-8.

MACKENZIE, A. & WADSWORTH, R.M. (2003). Extracellular L-arginine is required for optimal NO synthesis by eNOS and iNOS in the rat mesenteric artery wall. *Br J Pharmacol*, **139,** 1487-97.

MANIE, S., KONSTANTINOVA, I., BREITTMAYER, J.P., FERRUA, B. & SCHAFFAR, L. (1991). Effects of long duration spaceflight on human T lymphocyte and monocyte activity. *Aviat Space Environ Med*, **62,** 1153-8.

MAO, Q.W., ZHANG, L.F. & MA, J. (1999). [Differentiated remodeling changes of medium-sized arteries from different body parts in tail-suspended rats and their reversibility]. *Space Med Med Eng (Beijing)*, **12,** 92-6.

MARRELLI, S.P., KHOROVETS, A., JOHNSON, T.D., CHILDRES, W.F. & BRYAN, R.M., JR. (1999). P2 purinoceptor-mediated dilations in the rat middle cerebral artery after ischemia-reperfusion. *Am J Physiol*, **276,** H33-41.

MARTEL, E., CHAMPEROUX, P., LACOLLEY, P., RICHARD, S., SAFAR, M. & CUCHE, J.L. (1996). Central hypervolemia in the conscious rat: a model of cardiovascular deconditioning. *J Appl Physiol*, **80,** 1390-6.

MAXWELL, A.J. (2002). Mechanisms of dysfunction of the nitric oxide pathway in vascular diseases. *Nitric Oxide*, **6,** 101-24.

MAYER, B. (2000). *Handbook of Experimental Pharmacology. Nitric oxide*. Berlin ; New York: Springer.

MAYER, B. & ANDREW, P. (1998). Nitric oxide synthases: catalytic function and progress towards selective inhibition. *Naunyn Schmiedebergs Arch Pharmacol*, **358,** 127-33.

MCCURDY, M.R., COLLERAN, P.N., MULLER-DELP, J. & DELP, M.D. (2000). Effects of fiber composition and hindlimb unloading on the vasodilator properties of skeletal muscle arterioles. *J Appl Physiol*, **89,** 398-405.

MCDONALD, K.S., DELP, M.D. & FITTS, R.H. (1992). Effect of hindlimb unweighting on tissue blood flow in the rat. *J Appl Physiol*, **72,** 2210-8.

MECK, J.V., REYES, C.J., PEREZ, S.A., GOLDBERGER, A.L. & ZIEGLER, M.G. (2001). Marked exacerbation of orthostatic intolerance after long- vs. short-duration spaceflight in veteran astronauts. *Psychosom Med*, **63,** 865-73.

MEDOW, M.S., STEWART, J.M., SANYAL, S., MUMTAZ, A., SICA, D. & FRISHMAN, W.H. (2008). Pathophysiology, diagnosis, and treatment of orthostatic hypotension and vasovagal syncope. *Cardiol Rev*, **16,** 4-20.

MICHEL, T. & LAMAS, S. (1992). Molecular cloning of constitutive endothelial nitric oxide synthase: evidence for a family of related genes. *J Cardiovasc Pharmacol*, **20 Suppl 12,** S45-9.

MIGEOTTE, P.F., PRISK, G.K. & PAIVA, M. (2003). Microgravity alters respiratory sinus arrhythmia and short-term heart rate variability in humans. *Am J Physiol Heart Circ Physiol*, **284,** H1995-2006.

MIKI, K., ODA, M., KAMIJYO, N., KAWAHARA, K. & YOSHIMOTO, M. (2004). Lumbar sympathetic nerve activity and hindquarter blood flow during REM sleep in rats. *J Physiol*, **557,** 261-71.

MOCHIZUKI, S., HIMI, N., MIYASAKA, T., NAKAMOTO, H., TAKEMOTO, M., HIRANO, K., TSUJIOKA, K., OGASAWARA, Y. & KAJIYA, F. (2002). Evaluation of basic performance and applicability of a newly developed in vivo nitric oxide sensor. *Physiol Meas*, **23,** 261-8.

MOCHIZUKI, S., MIYASAKA, T., GOTO, M., OGASAWARA, Y., YADA, T., AKIYAMA, M., NEISHI, Y., TOYODA, T., TOMITA, J., KOYAMA, Y., TSUJIOKA, K., KAJIYA, F., AKASAKA, T. & YOSHIDA, K. (2003). Measurement of acetylcholine-induced endothelium-derived nitric oxide in aorta using a newly developed catheter-type nitric oxide sensor. *Biochem Biophys Res Commun*, **306,** 505-8.

MONCADA, S. & HIGGS, E.A. (2006). Nitric oxide and the vascular endothelium. *Handb Exp Pharmacol***,** 213-54.

MONCADA, S., PALMER, R.M. & HIGGS, E.A. (1989). Biosynthesis of nitric oxide from L-arginine. A pathway for the regulation of cell function and communication. *Biochem Pharmacol*, **38,** 1709-15.

MOREY, E.R., SABELMAN, E.E., TURNER, R.T. & BAYLINK, D.J. (1979). A new rat model simulating some aspects of space flight. *Physiologist*, **22,** S23-4.

MOREY-HOLTON, E.R. & GLOBUS, R.K. (2002). Hindlimb unloading rodent model: technical aspects. *J Appl Physiol*, **92,** 1367-77.

MULVAGH, S.L., CHARLES, J.B., RIDDLE, J.M., REHBEIN, T.L. & BUNGO, M.W. (1991). Echocardiographic evaluation of the cardiovascular effects of short-duration spaceflight. *J Clin Pharmacol*, **31,** 1024-6.

MURTHY, G., WATENPAUGH, D.E., BALLARD, R.E. & HARGENS, A.R. (1994). Supine exercise during lower body negative pressure effectively simulates upright exercise in normal gravity. *J Appl Physiol*, **76,** 2742-8.

NATHAN, C. & XIE, Q.W. (1994). Regulation of biosynthesis of nitric oxide. *J Biol Chem*, **269,** 13725-8.

NELSON, E.S. & JULES, K. (2004). The microgravity environment for experiments on the International Space Station. *J Gravit Physiol*, **11,** 1-10.

NELSON, M.T. & QUAYLE, J.M. (1995). Physiological roles and properties of potassium channels in arterial smooth muscle. *Am J Physiol*, **268,** C799-822.

NICOGOSSIAN, A.E., SAWIN, C.F., HUNTOON, C., NICOGOSSIAN, A.E., HUNTOON, C.L. & POOL, S.L. (1994). In *Physiologic adaptation to spaceflight: Overall Physiologic Response to Spaceflight*. pp. 213-227. Philadelphia: Lea & Fibiger.

NIXON, J.V., MURRAY, R.G., BRYANT, C., JOHNSON, R.L., JR., MITCHELL, J.H., HOLLAND, O.B., GOMEZ-SANCHEZ, C., VERGNE-MARINI, P. & BLOMQVIST, C.G. (1979). Early cardiovascular adaptation to simulated zero gravity. *J Appl Physiol*, **46,** 541-8.

NORSK, P., CHRISTENSEN, N.J., BIE, P., GABRIELSEN, A., HEER, M. & DRUMMER, C. (2000). Unexpected renal responses in space. *Lancet*, **356,** 1577-8.

NORSK, P., DRUMMER, C., ROCKER, L., STROLLO, F., CHRISTENSEN, N.J., WARBERG, J., BIE, P., STADEAGER, C., JOHANSEN, L.B., HEER, M. & ET, A.L. (1995). Renal and endocrine responses in humans to isotonic saline infusion during microgravity. *J Appl Physiol*, **78,** 2253-9.

O'LEARY, D.S. (1991). Regional vascular resistance vs. conductance: which index for baroreflex responses? *Am J Physiol*, **260,** H632-7.

OGANOV, V.S., GRIGOR'EV, A.I., VORONIN, L.I., RAKHMANOV, A.S., BAKULIN, A.V., SCHNEIDER, V.S. & LEBLANC, A.D. (1992). [Bone mineral density in cosmonauts after flights lasting 4.5-6 months on the Mir orbital station]. *Aviakosm Ekolog Med*, **26,** 20-4.

OVERTON, J.M. & TIPTON, C.M. (1990). Effect of hindlimb suspension on cardiovascular responses to sympathomimetics and lower body negative pressure. *J Appl Physiol*, **68,** 355-62.

OVERTON, J.M., WOODMAN, C.R. & TIPTON, C.M. (1989). Effect of hindlimb suspension on VO2 max and regional blood flow responses to exercise. *J Appl Physiol*, **66,** 653-9.

PALMER, R.F. & LASSETER, K.C. (1975). Drug therapy. Sodium nitroprusside. *N Engl J Med*, **292,** 294-7.

PALMER, R.M., FERRIGE, A.G. & MONCADA, S. (1987). Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature*, **327,** 524-6.

PAPADOPOULOS, A. & DELP, M.D. (2003). Effects of hindlimb unweighting on the mechanical and structure properties of the rat abdominal aorta. *J Appl Physiol*, **94,** 439-45.

PAVY-LE TRAON, A., HEER, M., NARICI, M.V., RITTWEGER, J. & VERNIKOS, J. (2007). From space to Earth: advances in human physiology from 20 years of bed rest studies (1986-2006). *Eur J Appl Physiol*, **101,** 143-94.

PAVY-LE TRAON, A., VASSEUR, P., ARBEILLE, P., GUELL, A., BES, A. & GHARIB, C. (1995). Effects of 28-day head-down tilt with and without countermeasures on lower body negative pressure responses. *Aviat Space Environ Med*, **66,** 982-91.

PERHONEN, M.A., FRANCO, F., LANE, L.D., BUCKEY, J.C., BLOMQVIST, C.G., ZERWEKH, J.E., PESHOCK, R.M., WEATHERALL, P.T. & LEVINE, B.D. (2001). Cardiac atrophy after bed rest and spaceflight. *J Appl Physiol*, **91,** 645-53.

PETERSON, T.E., GUICCIARDI, M.E., GULATI, R., KLEPPE, L.S., MUESKE, C.S., MOOKADAM, M., SOWA, G., GORES, G.J., SESSA, W.C. & SIMARI, R.D. (2003). Caveolin-1 can regulate vascular smooth muscle cell fate by switching platelet-derived growth factor signaling from a proliferative to an apoptotic pathway. *Arterioscler Thromb Vasc Biol*, **23,** 1521-7.

PLATTS, S.H., SHI, S.J. & MECK, J.V. (2006). Akathisia with combined use of midodrine and promethazine. *Jama*, **295,** 2000-1.

POLLOCK, J.S., FORSTERMANN, U., MITCHELL, J.A., WARNER, T.D., SCHMIDT, H.H., NAKANE, M. & MURAD, F. (1991). Purification and characterization of particulate endothelium-derived relaxing factor synthase from cultured and native bovine aortic endothelial cells. *Proc Natl Acad Sci U S A*, **88,** 10480-4.

POWERS, J. & BERNSTEIN, D. (2004). The mouse as a model of cardiovascular adaptations to microgravity. *J Appl Physiol*, **97,** 1686-92.

PRECKEL, M.P., LEFTHERIOTIS, G., FERBER, C., DEGOUTE, C.S., BANSSILLON, V. & SAUMET, J.L. (1996). Effect of nitric oxide blockade on the lower limit of the cortical cerebral autoregulation in pentobarbital-anaesthetized rats. *Int J Microcirc Clin Exp*, **16,** 277-83.

PRICE, D.T., VITA, J.A. & KEANEY, J.F., JR. (2000). Redox control of vascular nitric oxide bioavailability. *Antioxid Redox Signal*, **2,** 919-35.

PRISBY, R.D., WILKERSON, K., SOKOYA, E.M., BRYAN, R.M., WILSON, E. & DELP, M.D. (2006). Endothelium-Dependent Vasodilation of Cerebral Arteries Is Altered With Simulated Microgravity Through Nitric Oxide Synthase and EDHF Mechanisms. *J Appl Physiol*.

PRISK, G.K. (2000). Microgravity and the lung. *J Appl Physiol*, **89,** 385-96.

PRISK, G.K., GUY, H.J., ELLIOTT, A.R. & WEST, J.B. (1994). Cardiopulmonary adaptation to weightlessness. *J Gravit Physiol*, **1,** P118-21.

PURDY, R.E., SARA, D., DUCKLES, S.P. & KRAUSE, D.N. (1997). Simulated microgravity impairs vascular contractility. *Proc West Pharmacol Soc*, **40,** 25-7.

PURDY, R.E. & STUPECKY, G.L. (1984). Characterization of the alpha adrenergic receptor properties of rabbit ear artery and thoracic aorta. *J Pharmacol Exp Ther*, **229,** 459-68.

PURDY, R.E., WILKERSON, M.K., HUGHSON, R.L., NORSK, P. & WATENPAUGH, D.E. (2003). The cardiovascular system in microgravity: symposium summary. *Proc West Pharmacol Soc*, **46,** 16-27.

PUTCHA, L., BERENS, K.L., MARSHBURN, T.H., ORTEGA, H.J. & BILLICA, R.D. (1999). Pharmaceutical use by U.S. astronauts on space shuttle missions. *Aviat Space Environ Med*, **70,** 705-8.

QUAYLE, J.M., MCCARRON, J.G., BRAYDEN, J.E. & NELSON, M.T. (1993). Inward rectifier K+ currents in smooth muscle cells from rat resistance-sized cerebral arteries. *Am J Physiol*, **265,** C1363-70.

QUAYLE, J.M., NELSON, M.T. & STANDEN, N.B. (1997). ATP-sensitive and inwardly rectifying potassium channels in smooth muscle. *Physiol Rev*, **77,** 1165-232.

RAMSDELL, C.D., MULLEN, T.J., SUNDBY, G.H., ROSTOFT, S., SHEYNBERG, N., ALJURI, N., MAA, M., MUKKAMALA, R., SHERMAN, D., TOSKA, K., YELLE, J., BLOOMFIELD, D., WILLIAMS, G.H. & COHEN, R.J. (2001). Midodrine prevents orthostatic intolerance associated with simulated spaceflight. *J Appl Physiol*, **90,** 2245-8.

RAUSCHENBACH, B.V., SOKOLSKIY, V.N., GURJIAN, A.A., NICOGOSSIAN, A.E., MOHLER, S.R., GAZENKO, O.G. & GRIGORYEV, A.I. (1993). In *Historical aspects of space exploration.* pp. 1-50. Washington, DC: American Institute of Aeronautics and Astronautics.

REES, D.D., PALMER, R.M. & MONCADA, S. (1989). Role of endothelium-derived nitric oxide in the regulation of blood pressure. *Proc Natl Acad Sci U S A*, **86,** 3375-8.

REES, D.D., PALMER, R.M., SCHULZ, R., HODSON, H.F. & MONCADA, S. (1990). Characterization of three inhibitors of endothelial nitric oxide synthase in vitro and in vivo. *Br J Pharmacol*, **101,** 746-52.

RESCHKE, M.F., HARM, D.L., PARKER, D.E., SANDOZ, G.R., HOMICK, J.L., VANDERPLOEG, J.M., NICOGOSSIAN, A.E., HUNTOON, C.L. & POOL, S.L. (1994). In *Physiologic adaptation to spaceflight: Space motion sickness*. pp. 228-260. Philadelphia: Lea & Fibiger.

RILEY, D.A. (1999). Is skeletal muscle ready for long-term spaceflight and return to gravity? *Adv Space Biol Med*, **7,** 31-48.

RILEY, D.A. & ELLIS, S. (1983). Research on the adaptation of skeletal muscle to hypogravity: past and future directions. *Adv Space Res*, **3,** 191-7.

ROBERTSON, B.E., SCHUBERT, R., HESCHELER, J. & NELSON, M.T. (1993). cGMP-dependent protein kinase activates Ca-activated K channels in cerebral artery smooth muscle cells. *Am J Physiol*, **265,** C299-303.

ROBERTSON, D. & DAVIS, T.L. (1995). Recent advances in the treatment of orthostatic hypotension. *Neurology*, **45,** S26-32.

ROBERTSON, D., SHANNON, J.R., BIAGGIONI, I., ERTL, A.C., DIEDRICH, A., CARSON, R., FURLAN, R., JACOB, G. & JORDAN, J. (2000). Orthostatic intolerance and the postural tachycardia syndrome: genetic and environment pathophysiologies. Neurolab Autonomic Team. *Pflugers Arch*, **441,** R48-51.

ROER, R.D. & DILLAMAN, R.M. (1990). Bone growth and calcium balance during simulated weightlessness in the rat. *J Appl Physiol*, **68,** 13-20.

ROER, R.D. & DILLAMAN, R.M. (1994). Decreased femoral arterial flow during simulated microgravity in the rat. *J Appl Physiol*, **76,** 2125-9.

ROTHE, C.F. (1983). Reflex control of veins and vascular capacitance. *Physiol Rev*, **63,** 1281-342.

ROWELL, L.B. (1993). Human Cardiovascular Control. *New York. Oxford University Press*.

RUBANYI, G.M. (1991). Endothelium-derived relaxing and contracting factors. *J Cell Biochem*, **46,** 27-36.

RUBLE, S.B., VALIC, Z., BUCKWALTER, J.B., TSCHAKOVSKY, M.E. & CLIFFORD, P.S. (2002). Attenuated vascular responsiveness to noradrenaline release during dynamic exercise in dogs. *J Physiol*, **541,** 637-44.

RUMMEL, J.A., SAWIN, C.F., MICHEL, E.L., BUDERER, M.C. & THORNTON, W.T. (1975). Exercise and long duration speceflight through 84 days. *J Am Med Womens Assoc*, **30,** 173-87.

SAIJONMAA, O., RISTIMAKI, A. & FYHRQUIST, F. (1990). Atrial natriuretic peptide, nitroglycerine, and nitroprusside reduce basal and stimulated endothelin production from cultured endothelial cells. *Biochem Biophys Res Commun*, **173,** 514-20.

SALVEMINI, D., MISKO, T.P., MASFERRER, J.L., SEIBERT, K., CURRIE, M.G. & NEEDLEMAN, P. (1993). Nitric oxide activates cyclooxygenase enzymes. *Proc Natl Acad Sci U S A*, **90,** 7240-4.

SALVEMINI, D., SEIBERT, K., MASFERRER, J.L., MISKO, T.P., CURRIE, M.G. & NEEDLEMAN, P. (1994). Endogenous nitric oxide enhances prostaglandin production in a model of renal inflammation. *J Clin Invest*, **93,** 1940-7.

SANDLER, H., GOLDWATER, D.J., POPP, R.L., SPACCAVENTO, L. & HARRISON, D.C. (1985). Beta blockade in the compensation for bed-rest cardiovascular deconditioning: physiologic and pharmacologic observations. *Am J Cardiol*, **55,** 114D-119D.

SANDNER, P., KORNFELD, M., RUAN, X., ARENDSHORST, W.J. & KURTZ, A. (1999). Nitric oxide/cAMP interactions in the control of rat renal vascular resistance. *Circ Res*, **84,** 186-92.

SANGHA, D.S., HAN, S. & PURDY, R.E. (2001). Simulated microgravity upregulates an endothelial vasoconstrictor prostaglandin. *J Appl Physiol*, **91,** 789-96.

SANGHA, D.S., VAZIRI, N.D., DING, Y., HAN, S., ALEM, N., CHEN, Y.A. & PURDY, R.E. (1999). Simulated microgravity impairs vascular contractility: role of nitric oxide-dependent vasodilator mechanisms. *Proc West Pharmacol Soc*, **42,** 5-7.

SANGHA, D.S., VAZIRI, N.D., DING, Y. & PURDY, R.E. (2000). Vascular hyporesponsiveness in simulated microgravity: role of nitric oxide-dependent mechanisms. *J Appl Physiol*, **88,** 507-17.

SAUSBIER, M., SCHUBERT, R., VOIGT, V., HIRNEISS, C., PFEIFER, A., KORTH, M., KLEPPISCH, T., RUTH, P. & HOFMANN, F. (2000). Mechanisms of NO/cGMP-dependent vasorelaxation. *Circ Res*, **87,** 825-30.

SAWIN, C.F., BAKER, E. & BLACK, F.O. (1998). Medical investigations and resulting countermeasures in support of 16-day Space Shuttle missions. *J Gravit Physiol*, **5,** 1-12.

SAYET, I., NEUILLY, G., MIRONNEAU, J. & MIRONNEAU, C. (1995). Influence of spaceflight, hindlimb suspension, and venous occlusion on alpha 1-adrenoceptors in rat vena cava. *J Appl Physiol*, **78,** 1882-8.

SCHEPENS, J., CUPPEN, E., WIERINGA, B. & HENDRIKS, W. (1997). The neuronal nitric oxide synthase PDZ motif binds to -G(D,E)XV* carboxyterminal sequences. *FEBS Lett*, **409,** 53-6.

SCHLOSSMANN, J., AMMENDOLA, A., ASHMAN, K., ZONG, X., HUBER, A., NEUBAUER, G., WANG, G.X., ALLESCHER, H.D., KORTH, M., WILM, M., HOFMANN, F. & RUTH, P. (2000). Regulation of intracellular calcium by a signalling complex of IRAG, IP3 receptor and cGMP kinase Ibeta. *Nature*, **404,** 197-201.

SCHNEIDER, V.S., LEBLANC, A.D., TAGGART, L.C., NICOGOSSIAN, A.E., HUNTOON, C.L. & POOL, S.L. (1994). In *Physiologic adaptation to spaceflight: Bone and Mineral Metabolism*. pp. 327-333. Philadelphia: Lea & Fibiger.

SCHONDORF, R., BENOIT, J. & STEIN, R. (2001). Cerebral autoregulation in orthostatic intolerance. *Ann N Y Acad Sci*, **940,** 514-26.

SCHRAGE, W.G., WOODMAN, C.R. & LAUGHLIN, M.H. (2000). Hindlimb unweighting alters endothelium-dependent vasodilation and ecNOS expression in soleus arterioles. *J Appl Physiol*, **89,** 1483-90.

SESSA, W.C. (1994). The nitric oxide synthase family of proteins. *J Vasc Res*, **31,** 131-43.

SHAUL, P.W., SMART, E.J., ROBINSON, L.J., GERMAN, Z., YUHANNA, I.S., YING, Y., ANDERSON, R.G. & MICHEL, T. (1996). Acylation targets endothelial nitric-oxide synthase to plasmalemmal caveolae. *J Biol Chem*, **271,** 6518-22.

SHEYS, E.M. & GREEN, R.D. (1972). A quantitative study of alpha adrenergic receptors in the spleen and aorta of the rabbit. *J Pharmacol Exp Ther*, **180,** 317-25.

SHI, S.J., SOUTH, D.A. & MECK, J.V. (2004). Fludrocortisone does not prevent orthostatic hypotension in astronauts after spaceflight. *Aviat Space Environ Med*, **75,** 235-9.

SHOEMAKER, J.K., HOGEMAN, C.S., SILBER, D.H., GRAY, K., HERR, M. & SINOWAY, L.I. (1998). Head-down-tilt bed rest alters forearm vasodilator and vasoconstrictor responses. *J Appl Physiol*, **84,** 1756-62.

SHOUKAS, A.A. & SAGAWA, K. (1971). Total systemic vascular compliance measured as incremental volume-pressure ratio. *Circ Res*, **28,** 277-89.

SILVERSTEIN, M.D., SINGER, D.E., MULLEY, A.G., THIBAULT, G.E. & BARNETT, G.O. (1982). Patients with syncope admitted to medical intensive care units. *JAMA*, **248,** 1185-9.

SIMANONOK, K.E. & CHARLES, J.B. (1994). Space sickness and fluid shifts: a hypothesis. *J Clin Pharmacol*, **34,** 652-63.

SMIRNOV, S.V., BECK, R., TAMMARO, P., ISHII, T. & AARONSON, P.I. (2002). Electrophysiologically distinct smooth muscle cell subtypes in rat conduit and resistance pulmonary arteries. *J Physiol*, **538,** 867-78.

SMITH, S.M., WASTNEY, M.E., MORUKOV, B.V., LARINA, I.M., NYQUIST, L.E., ABRAMS, S.A., TARAN, E.N., SHIH, C.Y., NILLEN, J.L., DAVIS-STREET, J.E., RICE, B.L. & LANE, H.W. (1999). Calcium metabolism before, during, and after a 3-mo spaceflight: kinetic and biochemical changes. *Am J Physiol*, **277,** R1-10.

SONNENFELD, G. (1999). Space flight, microgravity, stress, and immune responses. *Adv Space Res*, **23,** 1945-53.

SOUTHAN, G.J. & SZABO, C. (1996). Selective pharmacological inhibition of distinct nitric oxide synthase isoforms. *Biochem Pharmacol*, **51,** 383-94.

STANDEN, N.B. & QUAYLE, J.M. (1998). K+ channel modulation in arterial smooth muscle. *Acta Physiol Scand*, **164,** 549-57.

STAUNTON, M., DREXLER, C., SCHMID, P.G., 3RD, HAVLIK, H.S., HUDETZ, A.G. & FARBER, N.E. (2000). Neuronal nitric oxide synthase mediates halothane-induced cerebral microvascular dilation. *Anesthesiology*, **92,** 125-32.

STEPHENSON, R.P. (1956). A modification of receptor theory. *Br J Pharmacol*, **11,** 379-93.

STRANDGAARD, S. & PAULSON, O.B. (1984). Cerebral autoregulation. *Stroke*, **15,** 413-6.

STREETEN, D.H. (1999). Orthostatic intolerance. A historical introduction to the pathophysiological mechanisms. *Am J Med Sci*, **317,** 78-87.

STUEHR, D.J. (1997). Structure-function aspects in the nitric oxide synthases. *Annu Rev Pharmacol Toxicol*, **37,** 339-59.

STUEHR, D.J., CHO, H.J., KWON, N.S., WEISE, M.F. & NATHAN, C.F. (1991). Purification and characterization of the cytokine-induced macrophage nitric oxide synthase: an FAD- and FMN-containing flavoprotein. *Proc Natl Acad Sci U S A*, **88,** 7773-7.

SU, Y., COUCH, M. & BLOCK, E.R. (1997). Substrate inhibition of nitric oxide synthase in pulmonary artery endothelial cells in culture. *Nitric Oxide*, **1,** 469-75.

SULZMAN, F.M. (1996). Life sciences space missions. Overview. *J Appl Physiol*, **81,** 3-6.

SUN, X.Q., YAO, Y.J., WU, X.Y., JIANG, S.Z., JIANG, C.L., CAO, X.S., HAO, W.Y. & QIAO, Z.L. (2002). Effect of lower body negative pressure against orthostatic intolerance induced by 21 days head-down tilt bed rest. *Aviat Space Environ Med*, **73,** 335-40.

TANAKA, K. (1996). Is nitric oxide really important for regulation of the cerebral circulation? Yes or no? *Keio J Med*, **45,** 14-27.

TANAKA, K., FUKUUCHI, Y., GOMI, S., MIHARA, B., SHIRAI, T., NOGAWA, S., NOZAKI, H. & NAGATA, E. (1993). Inhibition of nitric oxide synthesis impairs autoregulation of local cerebral blood flow in the rat. *Neuroreport*, **4,** 267-70.

TAYLOR, G.R. & DARDANO, J.R. (1983). Human cellular immune responsiveness following space flight. *Aviat Space Environ Med*, **54,** S55-9.

TAYLOR, G.R. & JANNEY, R.P. (1992). In vivo testing confirms a blunting of the human cell-mediated immune mechanism during space flight. *J Leukoc Biol*, **51,** 129-32.

TAYLOR, G.R., NEALE, L.S. & DARDANO, J.R. (1986). Immunological analyses of U.S. Space Shuttle crewmembers. *Aviat Space Environ Med*, **57,** 213-7.

TAYLOR, G.R. & NICOGOSSIAN, A.E. (1977). In *Medical microbial analysis of U.S. crewmembers*. pp. 69-85. Washington, DC: U.S. Government printing office.

THORNTON, W.E., HOFFLER, G.W., JOHNSTON, R.S. & DIETLEIN, L.F. (1977). In *Hemodynamic studies of the legs under weightlessness*. pp. 324-338. Washington, DC: Scientific and Technical Information Office, National Aeronautics and Space Administration.

TKACS, N.C. & THOMPSON, H.J. (2006). From bedside to bench and back again: research issues in animal models of human disease. *Biol Res Nurs*, **8,** 78-88.

TOBAL, N., ROUMY, J., HERAULT, S., FOMINA, G. & ARBEILLE, P. (2001). Doppler measurement of cerebral and lower limb flow during a lower body negative pressure test for predicting orthostatic intolerance. *J Ultrasound Med*, **20,** 1207-17.

TRACEY, W.R., POLLOCK, J.S., MURAD, F., NAKANE, M. & FORSTERMANN, U. (1994). Identification of an endothelial-like type III NO synthase in LLC-PK1 kidney epithelial cells. *Am J Physiol*, **266,** C22-8.

TURNER, R.T. (2000). Invited review: what do we know about the effects of spaceflight on bone? *J Appl Physiol*, **89,** 840-7.

VALLANCE, P., LEONE, A., CALVER, A., COLLIER, J. & MONCADA, S. (1992). Accumulation of an endogenous inhibitor of nitric oxide synthesis in chronic renal failure. *Lancet*, **339,** 572-5.

VAN ZWIETEN, P.A. & DOODS, H.N. (1995). Muscarinic receptors and drugs in cardiovascular medicine. *Cardiovasc Drugs Ther*, **9,** 159-67.

VAZIRI, N.D. (2003). Nitric oxide in microgravity-induced orthostatic intolerance: relevance to spinal cord injury. *J Spinal Cord Med*, **26,** 5-11.

VAZIRI, N.D., DING, Y., SANGHA, D.S. & PURDY, R.E. (2000). Upregulation of NOS by simulated microgravity, potential cause of orthostatic intolerance. *J Appl Physiol*, **89,** 338-44.

VERBANCK, S., LARSSON, H., LINNARSSON, D., PRISK, G.K., WEST, J.B. & PAIVA, M. (1997). Pulmonary tissue volume, cardiac output, and diffusing capacity in sustained microgravity. *J Appl Physiol*, **83,** 810-6.

VERNIKOS, J. & CONVERTINO, V.A. (1994). Advantages and disadvantages of fludrocortisone or saline load in preventing post-spaceflight orthostatic hypotension. *Acta Astronaut*, **33,** 259-66.

VERNIKOS, J., DALLMAN, M.F., VAN LOON, G. & KEIL, L.C. (1991). Drug effects on orthostatic intolerance induced by bedrest. *J Clin Pharmacol*, **31,** 974-84.

VIDEBAEK, R. & NORSK, P. (1997). Atrial distension in humans during microgravity induced by parabolic flights. *J Appl Physiol*, **83,** 1862-6.

WADSWORTH, R., STANKEVICIUS, E. & SIMONSEN, U. (2006). Physiologically relevant measurements of nitric oxide in cardiovascular research using electrochemical microsensors. *J Vasc Res*, **43,** 70-85.

WANG, Y., NEWTON, D.C., ROBB, G.B., KAU, C.L., MILLER, T.L., CHEUNG, A.H., HALL, A.V., VANDAMME, S., WILCOX, J.N. & MARSDEN, P.A. (1999). RNA diversity has profound effects on the translation of neuronal nitric oxide synthase. *Proc Natl Acad Sci U S A*, **96,** 12150-5.

WANG, Y.X., POON, K.S., RANDALL, D.J. & PANG, C.C. (1993). Endothelium-derived nitric oxide partially mediates salbutamol-induced vasodilatations. *Eur J Pharmacol*, **250,** 335-40.

WATENPAUGH, D.E., BALLARD, R.E., SCHNEIDER, S.M., LEE, S.M., ERTL, A.C., WILLIAM, J.M., BODA, W.L., HUTCHINSON, K.J. & HARGENS, A.R. (2000). Supine lower body negative pressure exercise during bed rest maintains upright exercise capacity. *J Appl Physiol*, **89,** 218-27.

WATENPAUGH, D.E., BALLARD, R.E., STOUT, M.S., MURTHY, G., WHALEN, R.T. & HARGENS, A.R. (1994). Dynamic leg exercise improves tolerance to lower body negative pressure. *Aviat Space Environ Med*, **65,** 412-8.

WATENPAUGH, D.E. & HARGENS, A.R. (1996). The cardiovascular system in microgravity. *Handbook of Physiology: Environmental Physiology III: The Gravitational Environment. New York, NY. Oxford University Press*, **I,** 631-674.

WATENPAUGH, D.E., O'LEARY, D.D., SCHNEIDER, S.M., LEE, S.M., MACIAS, B.R., TANAKA, K., HUGHSON, R.L. & HARGENS, A.R. (2007). Lower body negative pressure exercise plus brief postexercise lower body negative pressure improve post-bed rest orthostatic tolerance. *J Appl Physiol*, **103,** 1964-72.

WATERS, W.W., ZIEGLER, M.G. & MECK, J.V. (2002). Postspaceflight orthostatic hypotension occurs mostly in women and is predicted by low vascular resistance. *J Appl Physiol*, **92,** 586-94.

WEST, J.B. (2000). Physiology in microgravity. *J Appl Physiol*, **89,** 379-84.

WEST, J.B., ELLIOTT, A.R., GUY, H.J. & PRISK, G.K. (1997). Pulmonary function in space. *JAMA*, **277,** 1957-61.

WHITE, R.P., VALLANCE, P. & MARKUS, H.S. (2000). Effect of inhibition of nitric oxide synthase on dynamic cerebral autoregulation in humans. *Clin Sci (Lond)*, **99,** 555-60.

WHITSON, P.A., CHARLES, J.B., WILLIAMS, W.J. & CINTRON, N.M. (1995). Changes in sympathoadrenal response to standing in humans after spaceflight. *J Appl Physiol*, **79,** 428-33.

WHITSON, P.A., PIETRZYK, R.A., MORUKOV, B.V. & SAMS, C.F. (2001). The risk of renal stone formation during and after long duration space flight. *Nephron*, **89,** 264-70.

WHITSON, P.A., PIETRZYK, R.A. & SAMS, C.F. (1999). Space flight and the risk of renal stones. *J Gravit Physiol*, **6,** P87-8.

WILKERSON, M.K., COLLERAN, P.N. & DELP, M.D. (2002). Acute and chronic head-down tail suspension diminishes cerebral perfusion in rats. *Am J Physiol Heart Circ Physiol*, **282,** H328-34.

WILKERSON, M.K., LESNIEWSKI, L.A., GOLDING, E.M., BRYAN, R.M., JR., AMIN, A., WILSON, E. & DELP, M.D. (2005). Simulated microgravity enhances cerebral artery vasoconstriction and vascular resistance through endothelial nitric oxide mechanism. *Am J Physiol Heart Circ Physiol*, **288,** H1652-61.

WILKERSON, M.K., MULLER-DELP, J., COLLERAN, P.N. & DELP, M.D. (1999). Effects of hindlimb unloading on rat cerebral, splenic, and mesenteric resistance artery morphology. *J Appl Physiol*, **87,** 2115-21.

WILLIAMS, D.R. (2003). The biomedical challenges of space flight. *Annu Rev Med*, **54,** 245-56.

WOODMAN, C.R., SCHRAGE, W.G., RUSH, J.W., RAY, C.A., PRICE, E.M., HASSER, E.M. & LAUGHLIN, M.H. (2001). Hindlimb unweighting decreases endothelium-dependent dilation and eNOS expression in soleus not gastrocnemius. *J Appl Physiol*, **91,** 1091-8.

WOODMAN, C.R., SEBASTIAN, L.A. & TIPTON, C.M. (1995). Influence of simulated microgravity on cardiac output and blood flow distribution during exercise. *J Appl Physiol*, **79,** 1762-8.

XIE, M.J., ZHANG, L.F., MA, J. & CHENG, H.W. (2005). Functional alterations in cerebrovascular K(+) and Ca(2+) channels are comparable between simulated microgravity rat and SHR. *Am J Physiol Heart Circ Physiol*, **289,** H1265-76.

XUE, C., POLLOCK, J., SCHMIDT, H.H., WARD, S.M. & SANDERS, K.M. (1994). Expression of nitric oxide synthase immunoreactivity by interstitial cells of the canine proximal colon. *J Auton Nerv Syst*, **49,** 1-14.

ZHANG, L.F. (2000). Microgravity induced cardiovascular deconditioning: peripheral effector mechanism hypothesis and gravity-based countermeasure. *J Gravit Physiol*, **7,** P135-6.

ZHANG, L.F. (2001). Vascular adaptation to microgravity: what have we learned? *J Appl Physiol*, **91,** 2415-30.

ZHANG, L.F., SUN, B., CAO, X.S., LIU, C., YU, Z.B., ZHANG, L.N., CHENG, J.H., WU, Y.H. & WU, X.Y. (2003). Effectiveness of intermittent -Gx gravitation in preventing deconditioning due to simulated microgravity. *J Appl Physiol*, **95,** 207-18.

ZHANG, L.N., ZHANG, L.F. & MA, J. (2001). Simulated microgravity enhances vasoconstrictor responsiveness of rat basilar artery. *J Appl Physiol*, **90,** 2296-305.

ZHANG, R., ZUCKERMAN, J.H., PAWELCZYK, J.A. & LEVINE, B.D. (1997). Effects of head-down-tilt bed rest on cerebral hemodynamics during orthostatic stress. *J Appl Physiol*, **83,** 2139-45.

APPENDICES

APPENDIX 1 – UBC ANIMALS CARE PROTOCOL APPROVAL CERTIFICATE

THE UNIVERSITY OF BRITISH COLUMBIA

ANIMAL CARE CERTIFICATE

Application Number: A05-1691

Investigator or Course Director: Catherine C. Pang

Department: Pharmacology & Therapeutics

Animals: Rats Sprague-Dawley 200

Start Date: **April 1, 2006** **Approval Date:** **April 10, 2006**

Funding Sources:

Funding Agency: Canadian Space Agency

Funding Title: An integrated investigation into the adaptation of arterial and venous function following simulated microgravity in rats

Unfunded title: N/A

The Animal Care Committee has examined and approved the use of animals for the above experimental project.

APPENDIX 2 – DIAGRAM OF CUSTOM-BUILT APPARATUS FOR MEASUREMENT OF NITRIC OXIDE CONCENTRATION IN ISOLATED ARTERY PERFUSATE