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**TEMPERATURE EFFECTS ON HUMAN LEPTIN PHYSIOLOGY: POSSIBLE
IMPLICATIONS FOR THE REGULATION OF BODY COMPOSITION**

A thesis submitted in (partial) fulfilment of the requirements for the award of the degree

Doctor of Philosophy

from

The University of Wollongong

Annerieke Zeyl, M. Sc

Department of Biomedical Science

2006

CERTIFICATION

I, Annerieke Zeyl, declare that this thesis, submitted in partial fulfilment of the requirements for the award of Doctor of Philosophy, in the Department of Biomedical Sciences, University of Wollongong, is wholly my own work unless otherwise referenced or acknowledged. The document has not been submitted for qualifications at any other academic institution.

Annerieke Zeyl

Date _____

TEMPERATURE EFFECTS ON HUMAN LEPTIN PHYSIOLOGY: POSSIBLE IMPLICATIONS FOR THE REGULATION OF BODY COMPOSITION

ABSTRACT

In this study evidence has been sought for physiologically-significant, direct, local effects of reduced subcutaneous adipose tissue temperature on leptin as a possible mechanism for body composition regulation in response to environmental temperature. Twelve healthy males participated in 15 repeated cold-water immersions (study 1), human subcutaneous adipose tissue fragments ($n=7$) were incubated at 27°, 32° and 37°C (study 2) and fourteen healthy subjects underwent two cold-water immersions with and without the beta-adrenergic antagonist propranolol (study 3).

The first series of *in vivo* immersions were conducted over 15 days (60-90 min at 18°C). Acute cold exposure suppressed plasma leptin concentration (25 min: -14%, 60 min: -22%, $P<0.05$), whilst repeated cold-water immersion was associated with an increase of plasma leptin concentration relative to test day 1 (+19% day 8, +13% day 15, overall $P<0.05$). Leptin secretion *in vitro* decreased 3.7-fold as the incubation temperature decreased from 37° to 27°C ($P<0.05$). In a compartmental model of leptin turnover *in vivo*, the measured (local) temperature effect on leptin secretion *in vitro* was more than able to account for the observed cold-induced decrease in leptin concentration *in vivo*. In the second series, of cold-water immersions beta-adrenergic blockade by propranolol, which almost completely abolished cold induced release of non-esterified fatty acids ($P<0.05$), did not prevent the cold-induced decrease in plasma leptin concentrations ($P>0.05$). This latter result suggests that the sympathetic nervous system may be less important in cold-induced regulation of plasma leptin concentration than previously assumed.

The presented studies provide several lines of evidence for a role of leptin in energy balance regulation in response to environmental temperature. It is concluded that that local and direct effects of reduced subcutaneous adipose tissue temperature may be a more important contributor to the acute effects observed *in vivo*, than the sympathetically-mediated suppression of leptin secretion.

Publications arising from this thesis

1. Zeyl, A., Stocks, J.M., Taylor, N.A.S. and Jenkins, A.B. (2004). Interactions between temperature and human leptin physiology *in vivo* and *in vitro*. *European Journal of Applied Physiology and Occupational Physiology* 92: 571-578.
2. Zeyl, A., Haley, C.D., Thoicharoen, P., Welschen, L.M.C., Sinnema, N.C.A., Taylor, N.A.S., and Jenkins, A.B. Increased post-immersion afterdrop during beta-adrenergic blockade. *The 10th International Conference on Environmental Ergonomics September 23-27, 2002 Fukuoka, Japan*.
3. Zeyl, A., Haley, C.D., Thoicharoen, P., Taylor N.A.S. and Jenkins, A.B. Beta-adrenergic blockade does not prevent cold-induced decrease in circulating leptin. *9th International Congress on Obesity, August 24-29, 2002, Sao Paolo, Brazil*.
4. Zeyl, A., Stocks, J.M., Taylor, N.A.S., and Jenkins, A.B. (2001). Cold-induced decreases in human, circulating leptin, and in the subcutaneous adipose leptin secretion rate. *Proceedings of the Australian Physiological and Pharmacological Society. 32(1: supplement 1):188P. Associated Conference: International Thermal Physiology Symposium. September 2nd- 6th, 2001. Wollongong, Australia*.
5. Zeyl, A., Lim-Fraser, M., Lapsys, N.M., Cooney, G., Taylor, N.A.S., and Jenkins, A.B. (2000). Effects of temperature on metabolism and leptin secretion in human subcutaneous adipose tissue *in vitro*. *2000 Pre-Olympic Congress: International Congress on Sport Science, Sport Medicine and Physical Education, The Australasian Society for the Study of Obesity. September 7th-12th, 2000. Brisbane, Qld, Australia. P 99*.
6. Zeyl, A., Regan, J.M., Taylor, N.A.S., and Jenkins, A.B. (1998). Relationship between leptin levels and rectal temperature during cold-water immersion. *The Australian Society for the Study of Obesity. Gold Coast, Australia, October 24th-25th, 1998*.

7. Zeyl, A., Regan, J.M., Patterson, M.J., Taylor, N.A.S., and Jenkins, A.B. (1998). Plasma leptin: an apparent interaction with acute cold exposure. *Proceedings of the Australian Physiological and Pharmacological Society*. 29(2):63P.
8. Zeyl, A., J.M. Regan, M.P. Patterson, N.A.S. Taylor and A.B. Jenkins (1998). The effects of repeated cold-water exposure on plasma leptin concentration in humans. *Diabetologia* 41(S1):A219
9. Zeyl, A., Regan, J.M., Patterson, M.J., Taylor, N.A.S., and Jenkins, A.B. (1997). The effects of cold-water exposure on plasma leptin concentration in humans. *The Australian Society for the Study of Obesity*. Canberra, Australia, September 28th-29th, 1997.

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CHAPTER 1: INTRODUCTION AND HYPOTHESES

Obesity is in the media often portrayed as a ‘new’ disease and suggested to be the result of a modern society with an established fast food industry. However, it was already in 1936 that Du Bois noted that ‘overnutrition is prevalent in our country (United States of America) and obesity is one of the most common diseases’. He also remarked that while there was a wide public interest into the regulation of body weight, the scientific information was limited at that time. Using simple calculations, he demonstrated that energy homeostasis, maintaining body weight by matching energy intake on energy expenditure, is accurately regulated allowing less than 0.05% error (*i.e.* mismatch between energy intake and expenditure) over a twenty-year period of weight stability. However, the underlying mechanism of this tight regulation of body weight in normal and the disturbed regulation in obese and in ‘people with an endocrine disturbances’ remained to be discovered (Du Bois, 1936).

The regulation of food intake, energy expenditure and body weight has been the topic of numerous investigations in the last century. From these studies it is clear that genetic, physiological and behavioural factors are all involved in body weight regulation. Long-term energy imbalance results predominantly in changes in adipose tissue mass as protein and carbohydrates stores in the body are limited. Additionally, in the last decades various hormonal (*e.g.* insulin and leptin) and neural signals (*e.g.* neuropeptide-Y) have been identified in the underlying mechanisms of energy balance regulation (Jequier and Tappy, 1999; Woods *et al.*, 2000; Cupples, 2005). Despite these advances in scientific knowledge, a complete understanding of mechanisms involved in the regulation of human adipose tissue mass has remained elusive.

1.1 TEMPERATURE AND BODY WEIGHT

The concept that temperature may play a role in shaping variation in body mass, originates from the German biologist Karl Bergmann (1847). This concept, later known as Bergmann’s rule, states, “Within a polytypic warm-blooded species, the body size of the subspecies usually increases with decreasing mean temperature of it’s habit”. There is evidence that humans also follow the ecological rule of Bergmann such that those

individuals inhabiting colder regions are heavier than people living in warmer regions. Roberts analysed data collected in 1953 on body mass and mean annual temperature. His results demonstrate significant negative correlations between body mass and mean annual temperature and therefore agree with the concept that humans conform to Bergmann's rule (Robert, 1953; Roberts, 1978).

The relationship between mass and mean annual temperature is later confirmed by Katzmarzyk and Leonard (1998). They compared Robert's results with data published after 1953. While their results demonstrate similar relationships, it should be noted that the correlations and slopes of regression were lower than those found by Roberts (Mass: -0.59 vs. 0.27) and (BMI: 0.58 vs. 0.22). It is hypothesised that the weaker relationships result from secular trends on body mass over the time period 1953 and 1998. Data of the above-described studies do not differentiate between the three different body compartments, however variations in adipose tissue seem to be the most likely contributor to climate-induced variations in body mass. Despite the confirmation by Katzmarzyk and Leonard of the relationship between environmental temperature and body weight, experimental investigations in this area are limited. Moreover, while the effects of temperature on energy balance were documented early on (Leblanc, 1957; Dauncey, 1981), an underlying mechanism that could possibly link changes in environmental temperature to human body composition regulation has only incidentally been addressed in the literature.

1.2 TEMPERATURE EFFECTS ON ENERGY BALANCE

Both humans and homeothermic animals exhibit typical metabolic responses when they are exposed to cold in order to maintain body temperature. Morphological, behavioural and physiological adaptations have been demonstrated to occur in terrestrial mammals (Feist and White, 1989). In larger, furred animals, the seasonal adjustments in insulation may result in maintenance of metabolic rate despite reduction in environmental temperature. Small mammals, which have a high mass-specific conductance, respond to cold by increasing energy expenditure by means of non-shivering thermogenesis, looking for shelter, as well going into torpor or hibernation. Furthermore, increases in food intake have been observed in order to compensate the increased energy demand during cold (Morrison, 1981; Collier *et al.*, 1989). Clearly, the

energy balance is significantly challenged during cold-exposure. And when the increased energy expenditure is not exactly matched by the increase energy intake, changes in body weight occur. Decreases in body weight of rodents have indeed been demonstrated during chronic cold exposure as the marked increased energy expenditure was not completely compensated by increases in food intake (Hori *et al.*, 2001).

1.3 WHITE ADIPOSE TISSUE

In higher organisms, excess energy intake is stored as triglycerides in adipose tissue. Adipose tissue is located either deep and around the internal organs or subcutaneously. In humans, the subcutaneous depot is the major storage area, where >60% of the total body fat mass is located (Ramsay, 1996). Subcutaneous adipose tissue serves both as an energy storage depot and as a thermal insulator. While the temperature of deep adipose tissue depots approximates core temperature, human subcutaneous adipose tissue temperature has, similar as skin temperature, a non-uniform distribution. Le Blanc (1954) demonstrated that observed variations in skin temperatures between and within humans can be explained by variations of the thickness of the underlying fat layer. Moreover, as a result of the insulating function of the subcutaneous adipose tissue layer, subcutaneous adipose tissue and skin temperatures are strongly influenced by environmental temperature, and vary across wide ranges especially during cold-exposure (Livingston *et al.*, 1987; Frim *et al.*, 1990; Webb, 1992). For example, Webb (1992) showed that the difference in temperature between two depots within the same individual was significantly greater at low environmental temperature (5.3°C at 15°C) than in a hot condition (1.5°C at 45°C). The consequences and interactions of these local temperature changes on local adipose tissue physiology and metabolism have however barely been addressed in the literature.

1.3.1 Adrenergic Innervation of White Adipose Tissue

White adipose tissue (WAT) is one of the few organs, together with blood vessels and sweat glands that have only sympathetic and no parasympathetic nerve supply. Bartness and Bamshad (1998) have extensively discussed both physiological and neuro-anatomical evidence of the sympathetic innervation of WAT. Physiological evidence for sympathetic innervation is derived from studies measuring

electrophysiological responses, investigating the impact of denervation on lipid metabolism and electrical stimulation of WAT. These studies have shown that the Sympathetic Nervous System (SNS) plays an essential role in the regulation of lipolysis, since denervation leads to an increased adipose tissue mass, while nerve and receptors stimulation increased the release of free fatty acids (FFA) and the level of circulating FFA. There are some neuro-anatomical studies that show direct and *en passant* innervation of adipocytes, while others only demonstrate vascular innervation in WAT. From these studies it can be concluded that while the exact nature of SNS innervation of WAT is unclear, it is a part of the general SNS outflow from the central nervous system.

1.4 LEPTIN

1.4.1 Leptin: a Product of White Adipose Tissue

While for a long time adipose tissue was considered to be a passive organ, in the last few decades it has become apparent that adipose tissue is an active metabolic and endocrine organ. This tissue expresses and secretes various proteins such as leptin, adiponectin, complement components, plasminogen activator inhibitor-1, proteins of the renin-angiotensin system and resistin (Kershaw and Flier, 2004). The current investigation has focused on leptin and its possible role in the regulation of body composition in response to environmental temperature changes for the following reasons. First of all, leptin is secreted in direct proportion to the amount of body fat stored and has been suggested to act as a negative feedback signal on local energy storage (Havel *et al.*, 1996). Secondly, leptin receptors in the brain have been shown to influence to both determinants of energy balance i.e. energy intake and expenditure (Zhang *et al.*, 1994; Campfield *et al.*, 1995; Halaas *et al.*, 1995). Finally, temperature effects on leptin have been demonstrated (Trayhurn *et al.*, 1995; Hardie *et al.*, 1996b; Ricci *et al.*, 2000).

1.4.2 The Discovery of Leptin

In late 1940's, it was discovered that a particular strain of mice developed obesity due to spontaneous gene mutation and this strain was therefore named the *ob/ob*

mice. Parabiotic experiments demonstrated that cross circulation between obese and lean mice altered eating behaviour and the metabolic rate of the *ob/ob* mice (Coleman, 1973). This finding suggested the existence of a circulating substance involved in the regulation of energy balance, of which the effects were increasing energy expenditure and decreasing appetite. These experiments were a scientific breakthrough in the metabolic research field. And extensive nutritional, physiological and biochemical studies with the *ob/ob* mice followed this discovery. However, the fundamental defect in the *ob/ob* mice remained elusive until 1994. Studies of Zhang *et al.* (1994) revealed that a gene mutation in the *ob/ob* mice results in a deficiency of an adipose tissue derived hormone that was named leptin. Their results further suggested that leptin plays a role in the signalling pathway from adipose tissue to the brain regarding the size of the body fat depot.

This discovery evoked numerous studies on the regulation and actions of leptin. It was demonstrated that the *ob* gene is predominantly expressed in WAT and the expression of *ob* mRNA and plasma leptin concentration are correlated with indexes of body fatness (Rosenbaum *et al.*, 1996). These latter findings supported the hypotheses that leptin provides a feedback signal to the hypothalamus indicating the size of fat stores. The main actions of leptin have been confirmed to be: 1) increasing energy expenditure and 2) suppression of appetite (decreasing food intake) together resulting in weight loss. However, additional involvement of leptin in various neuro-endocrine systems, immune function, haematopoiesis, angiogenesis, renal function and especially in the reproductive system have also been demonstrated (Laughlin and Yen, 1997; Houseknecht *et al.*, 1998; Trayhurn *et al.*, 1999). Additionally, it has been shown that the production of leptin is not limited to WAT alone but various other organs such as the stomach, placenta and brain also produce leptin. However, none of these sites seem to be able to produce leptin to a similar extent as white adipose tissue (Himms-Hagen, 1999).

Besides regulating of adiposity by binding to hypothalamic leptin receptors affecting energy expenditure and appetite, there is also evidence for direct effects of leptin on adipose tissue metabolism. For example, Zhang *et al.* (1999) demonstrated an inhibitory effect of leptin on glucose uptake in adipose tissue.

Finally while it is more rare than originally was assumed, a few human studies have identified leptin receptor mutation together with obesity (Clement *et al.*, 1996; Montague *et al.*, 1997). These studies demonstrated not only the existence of gene mutations in humans, but more importantly that leptin is also involved in the regulation of energy balance and body fat in man (Trayhurn *et al.*, 1999).

1.4.3 Temperature Effects on Leptin Physiology

Rodent studies were the first to demonstrate interactions between thermal stress and *ob* gene expression (Trayhurn *et al.*, 1995) and circulating leptin (Hardie *et al.*, 1996b). Trayhurn *et al.* found that acute cold-air exposure to (4°C), for as short as 2-4 h, greatly reduced *ob* gene expression in mice. While similar cold exposure for 24 h resulted in a 25% reduction in circulating leptin in lean Zucker rats (Hardie *et al.*, 1996b). Furthermore, other rodent studies show that administration of exogenous leptin during cold exposure reduces the decrease in T_c (Stehling *et al.*, 1997), and that immunoneutralisation of circulating leptin results in a decreased T_{re} (Martinez *et al.*, 1998). The acute effect of reduced ambient temperature on plasma leptin concentration has also been reported in humans. Ninety min of cold air (6.3°C) exposure significantly reduced plasma leptin concentration in five female subjects (Ricci *et al.*, 2000). Similar as Trayhurn *et al.* in the rodent studies, Ricci *et al.* suggested that this decrease in plasma leptin concentration results from sympathetic nervous system activation during cold exposure.

1.4.4 Leptin and Energy Balance During Cold Exposure

Studies in hibernating animals suggest that leptin may be involved in energy balance regulation and behavioural responses during cold exposure (Gavrilova *et al.*, 1999; Kronfeld-Schor *et al.*, 2000; Nieminen *et al.*, 2001). In raccoon dogs in the months preceding hibernating (August-October) leptin levels increase in parallel with increases of their energy stores. However, when the energy stores have reached their maximum (up to 25% of body mass is stored subcutaneously) leptin levels tend to sharply decrease without any changes in body mass and remained suppressed during hibernation. In contrast, in the blue foxes, a non-hibernating animal, plasma leptin concentration increased during the second half of winter, again independent of body

mass changes. These dissociations between adiposity and leptin supports a role for leptin in energy balance regulation during cold-exposure. However, these results also indicate that the role of leptin differs among animal species and depends on the animal's energy stores (Nieminen *et al.*, 2001).

Humans do not have the same effector systems that are important in other animals (*e.g.* brown adipose tissue), do not exhibit the same type of behavioural responses (shivering rather than torpor and hibernation) and lack substantial endogenous insulation (*e.g.* fur) distal to their subcutaneous fat depots. In humans, the predominantly subcutaneously stored adipose tissue has a high *ob* gene expression and is, due to the lack of fur, subjected to a wider range of temperature than most other tissues. Similar as in animals the relationship between leptin and body fatness undergoes short-term dissociation during cold-exposure (Boden *et al.*, 1996; Ricci *et al.*, 2000). Therefore, it could be suggested that leptin may also play a role in energy balance regulation during cold-exposure in humans.

1.4.5 Beta-adrenergic Regulation of Leptin

Both human and rodents studies have shown inhibitory effects of beta-adrenoreceptor agonists on both circulating leptin concentration and *ob* mRNA expression and (Trayhurn *et al.*, 1995; Giacobino, 1996; Deng *et al.*, 1997; Pinkney *et al.*, 1998; Ricci and Fried, 1999). Therefore, the sympathetic nervous system, via the beta-adrenergic pathway, is assumed to be the major regulator of leptin production (Rayner and Trayhurn, 2001). However, the specific beta-receptor involvement seems to differ between human and rodents. In rodents, it is well established that all three beta-receptors are involved in the regulation of leptin (Giacobino, 1996; Deng *et al.*, 1997). However, whether the beta₃-adrenergic receptor is also involved in human leptin regulation is questionable. Several studies have been conducted to investigate whether a relationship exist between polymorphism of the beta₃-adrenergic receptor and plasma leptin concentration. These studies showed no associations between the Trp64Arg variant of the beta₃-adrenergic receptor and serum leptin concentrations (Janssen *et al.*, 1998; Snitker *et al.*, 1998; Stangl *et al.*, 2000; Hsueh *et al.*, 2001).

Additionally, an *in vitro* study investigated the specific involvement of three beta-receptors in the regulation of leptin secretion from human adipocytes. This study demonstrates that the different selective beta-adrenergic agonists used in this study increased glycerol release into the culture media in a dose and time dependent manner, indicating that all three beta-adrenergic receptor types are involved in the regulation of lipolysis. In contrast, stimulation of the different beta-adrenergic receptors had differential effects on leptin secretion. Isoproterenol, a non-selective agonist, markedly reduced leptin secretion. Smaller effects were found for dobutamine (beta₁-selective) and fenoterol (beta₂ selective). However, both beta₃ selective agonists (BRL 37344 and CGP 12177) had no effect on leptin secretion (Scriba *et al.*, 2000).

Finally, both infusion of isoprenaline and epinephrine significantly decreases circulating leptin in humans (Pinkney *et al.*, 1998; Couillard *et al.*, 2002). However, no acute or long-term effect of administration of two beta₃ adrenoreceptors agonists (L-796568 and CL316243, respectively) on plasma leptin concentrations were found (Weyer *et al.*, 1998; van Baak *et al.*, 2002). Therefore, it can be concluded that in humans, beta-adrenergic regulation of leptin is mainly through beta₁ and beta₂ receptors stimulation and not via beta₃ receptors.

1.4.6 Local Mechanisms of Leptin Regulation

While sympathetic activation is often considered as the underlying mechanism in leptin physiology during the cold, direct and local effects of temperature on human omental adipose tissue leptin secretion have been demonstrated (Peino *et al.*, 2000). Tissue fragments of omental adipose tissue were incubated at three different temperatures (32, 34.5 and 37°C) and subsequent analysis of leptin secretion into the media and *ob* gene expression were performed. Peino *et al.* demonstrated that leptin secretion was significantly reduced at lower temperatures. However, no effects of temperature on *ob* mRNA expression in adipose tissue were found. As similar results were obtained in growth hormone secretion from GH3 cells, the authors concluded that reduced leptin secretion at lower temperature results from “non-specific” actions and that these therefore may have no further physiological implications. However, whether or not a non-specific action is of physiological relevance would depend on the contribution of these direct, local effects to the *in vivo* observed effects. Furthermore, as

both human omental adipose tissue and GH3 cells are not likely to be exposed to significant temperature fluctuations, data from similar studies using subcutaneous adipose tissue may be of greater physiological relevance.

1.5 SUMMARY

Figure 1.1 summarises currently established relationships and pathways suggested to be involved in the regulation of body weight in response to environmental temperature and the hypothetical involvement of leptin via local modification of adipose tissue temperatures.

Energy balance is substantially challenged during cold exposure as energy expenditure is increased to maintain body temperature which needs to be matched exactly by energy intake otherwise body weight changes occur. Subcutaneous adipose tissue serves not only as an energy store but also as a thermal insulator and its temperature is substantially affected by environmental temperature. Leptin, a hormone predominantly secreted by adipose tissue, has been shown to be involved in the regulation of energy balance and thus of adiposity. Leptin secretion and *ob* gene expression has been shown to be higher in subcutaneous than in omental adipose tissue. Together these results may indicate a role for leptin in the regulation of body weight in response to environmental temperature changes. Cold exposure has been shown to decrease both plasma leptin concentration and *ob* mRNA expression. However, the underlying mechanisms and physiological implications of these cold-induced reductions have been barely addressed.

1.6 AIMS AND HYPOTHESES

General: This project aims to investigate mechanisms possibly involved in the regulation of adiposity in response to environmental temperature.

Specifically: This project aims to investigate mechanisms involved in the cold-induced changes in plasma leptin concentration.

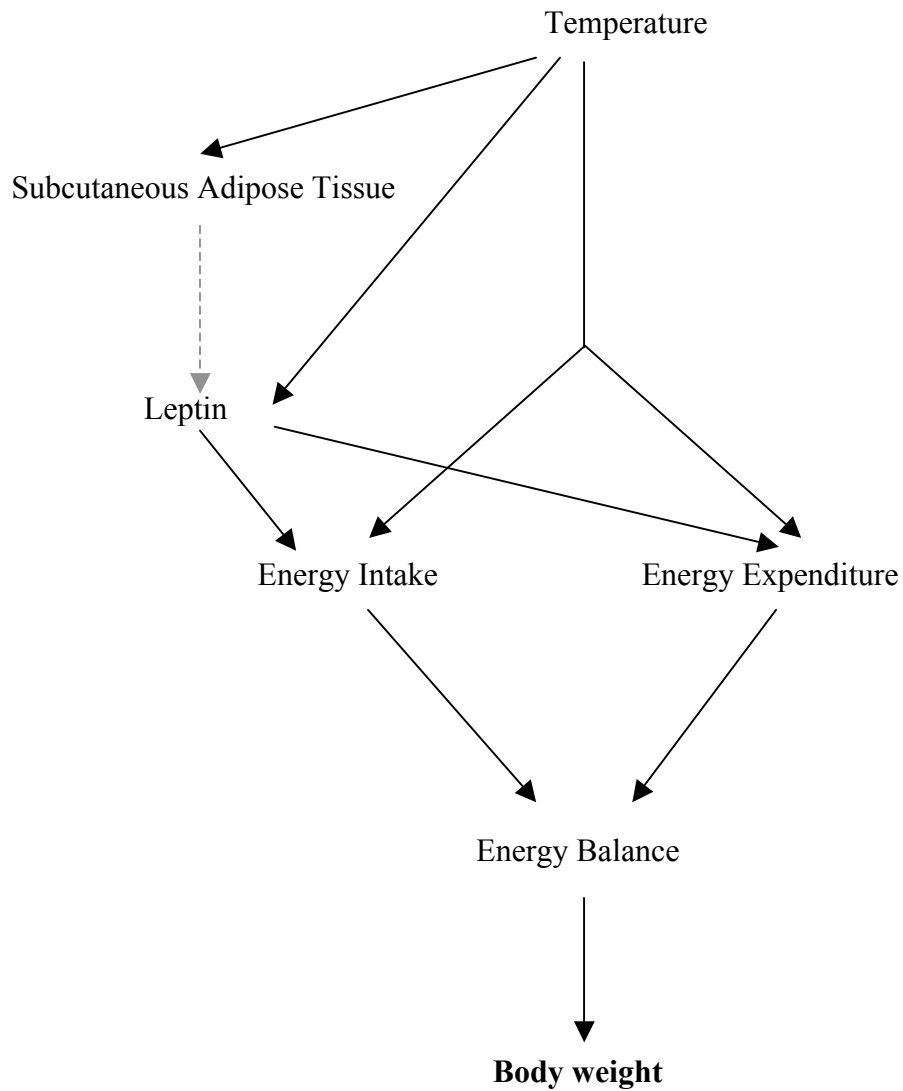


Figure 1.1: Hypothetical model of possible pathways by which environmental temperature may be involved in the regulation of body weight. Black solid lines represent individual demonstrated relationships or effects. Grey dotted line indicates hypothesised local and direct effect of modified of subcutaneous adipose tissue temperature on leptin physiology.

It was hypothesized that:

- During cold exposure, local temperature effects within the adipose tissue self are an important mechanism underlying the observed changes in circulating leptin.

This hypothesis was investigated by conducting three experiments with the aims to demonstrate the following:

1. By decreasing body temperatures, acute cold exposure, results in a decrease in human circulating leptin.
2. A mathematical model, derived from *in vitro* data of the effects of incubation temperature in leptin secretion by cultured human adipose tissue fragments, can be used to clarify the decreases in circulating leptin in cold exposed subjects.
3. Oral administration of propranolol (80 mg), a β_1 and β_2 -adrenergic antagonist, does not prevent cold-induced decreases in plasma leptin concentration.

These aims were tested in:

A series of fifteen daily repeated cold-water immersions experiments, in which subjects were immersed in 18°C water. On day 1, 8 and 15 body temperatures and cardiac frequency were continuously monitored. Blood samples were collected at various points in time prior to, during and after immersion and were assayed for plasma leptin, non-esterified fatty acids and glucose concentrations.

Tissue culture experiments in which human adipose tissue fragments were incubated at three different incubation temperatures (27, 32 and 37°C). Leptin secretion, glucose uptake and non-esterified fatty acids release were determined from medium samples collected at various points in time during the incubation period.

Finally, a second series of cold-water experiments, in which each subject underwent two separate trials one with and one without administration of beta adrenergic antagonist propranolol. Also in these experiments body temperatures and cardiac frequency were continuously monitored. Blood samples were collected during the

immersions and assayed for serum leptin, non-esterified fatty acids, and glucose concentrations. Additionally, forearm and skin blood flow were determined using venous-occlusion plethysmography and laser-Doppler velocimetry respectively.

CHAPTER 2: INTERACTIONS BETWEEN TEMPERATURE AND HUMAN LEPTIN PHYSIOLOGY *IN VIVO* AND *IN VITRO*.

2.1 INTRODUCTION

2.1.1 Environmental Temperature and Energy Balance

A complete understanding of the mechanism involved in the regulation of human adipose tissue mass has remained elusive. In this investigation evidence was sought for possible interactions between environmental temperature and energy balance in humans, via the local modification of adipose tissue temperatures and consequent effect on plasma leptin concentration.

Anecdotal evidence indicates that climatic conditions can modify both appetite and energy intake in humans (Westerterp-Plantenga, 1999). However, while there is some indication that humans living in colder regions tend to exhibit greater adiposity than people from warmer regions (Roberts, 1978; Katzmarzyk and Leonard, 1998), there is little experimental support for this relationship. Nevertheless, since subcutaneous adipose deposits fulfil the dual role of energy storage and thermal insulation, then climate-induced variations in body mass may reflect differences in both adiposity and cold stress, with the latter driving the former. As a consequence of its insulating function, and its proximity to the skin surface, the temperature of subcutaneous adipose tissue is strongly influenced by environmental temperature, and varies over a wide range particularly during cold exposure (Webb, 1992). Accordingly, the primary focus of this study was to investigate the possibility that environmental temperature may exert direct, local effects on leptin secretion from subcutaneous adipose tissues, and that these local effects may make a substantial supplementary contribution to the cold-induced reduction in circulating leptin *in vivo*. The herein presented data supports the former relationship.

2.1.2 Temperature Effects on Leptin Physiology

Leptin is predominantly secreted by adipose tissue and, via a feedback loop to hypothalamic receptors, it regulates adiposity through simultaneous effects on energy expenditure and appetite (Havel, 2000). There is evidence, at least in animals, for a direct effect of leptin on adipose tissue metabolism, via an inhibition of glucose uptake (Zhang *et al.*, 1999) and for an interaction between ambient temperature and both circulating leptin concentration and *ob* gene *mRNA* expression (Trayhurn *et al.*, 1995; Hardie *et al.*, 1996b; Bing *et al.*, 1998). For example, Trayhurn *et al.* (1995) found that cold-air exposure (4°C) for 2-18 h reduced *ob* gene expression in white adipose tissue of mice, while Moinat *et al.* (1995) observed reductions in the brown adipose tissue of rats (24 h at 6°C). Similar cold exposures have elicited a 25% reduction in circulating leptin in lean Zucker rats, but not in obese rats (Hardie *et al.*, 1996b). In humans, significant decreases in plasma leptin concentration (-14%, -17%, -22%) accompanying cold air exposures (6.3°C: 30, 60, and 90 min, respectively) have also been reported (Ricci *et al.*, 2000). Collectively, these observations led us to suspect that a relationship between environmental temperature and circulating leptin may modify energy expenditure and appetite, to determine relative adiposity. Indeed, Stehling *et al.* (1997) suggested that circulating leptin might even play a role in thermal homeostasis, after observing that the administration of recombinant leptin to rat pups attenuated the reduction in core temperature (T_c) associated with acute cold stress.

2.1.3 Mechanisms of Cold-induced Decrease in Circulating Leptin and *Ob* Gene Expression

Cold-induced decreases in *ob* gene expression and plasma leptin concentration have been suggested to result from sympathetic activation during cold exposure (Evans *et al.*, 1999; Ricci *et al.*, 2000; Rayner and Trayhurn, 2001). Recently, Peino *et al.* (2000) studied local temperature effects on leptin secretion from human omental adipose tissue, observing that low temperatures directly reduced leptin secretion rate. While these visceral tissues are unlikely to experience either frequent, or significant, temperature fluctuations *in vivo*, in adequately-clothed people, the larger subcutaneous adipose depots, on the other hand, can rapidly undergo 5°C changes during cold-air

exposure (Webb, 1992) and up to 10°C during cold-water immersion (Zeyl *et al.*, 2001). Therefore, it was postulated that a direct (local) affect of environmental temperature may also contribute to this cold-induced decrease in plasma leptin concentration.

No previous studies have reliably estimated the contribution of the sympathetic nervous system, or other mechanisms, to the cold-induced decrease in circulating leptin concentrations *in vivo*. While the results of *in vivo* sympathetic blockade of cold-exposed mice have been interpreted to support a predominant role for the sympathetic nervous system (Evans *et al.*, 1999), closer inspection of those data reveals that interpretation to be unconvincing. Thus, while not discounting a sympathetic role, it is believed that additional mechanisms should also be investigated.

Accordingly, it was hypothesised that acute cold exposure in humans may affect leptin secretion from subcutaneous adipose tissue, which in turn could modulate energy balance and adiposity. It was also of interest to evaluate the possibility of adaptation trends within these responses, since such adaptation is well established for other aspects of thermal physiology. To evaluate these possibilities, two human experiments were conducted. The first study investigated the effects of both acute and repeated cold-water immersion on subcutaneous tissue temperatures (T_{sub}) and circulating leptin concentration *in vivo*. The second study looked at the acute effects of incubation temperature on leptin secretion rate from human subcutaneous adipose tissue fragments *in vitro*. From these data, a compartmental model was derived, to further investigate the extent to which direct, local effects of reduced T_{sub} on leptin secretion rate could account for the *in vivo* observations of a cold-induced decrease in plasma leptin concentration.

2.2 METHODS

Both projects were approved by the Human Research Ethics Committee (University of Wollongong), with all subjects providing written informed consent.

2.2.1 Study 1: Cold-water Immersion:

2.2.1.1 Subjects

Twelve healthy, lean males [mass $72.2 \text{ kg} \pm 8.3$ (S.D.); height $1.75 \pm 0.07 \text{ m}$; body mass index $23.6 \text{ kg}\cdot\text{m}^{-2} \pm 2.0$] participated in 15 days of repeated cold-water immersion. Seven of these subjects also participated in a more extensive study, involving measurements of thermogenic responses, body-fluid compartment changes and hormonal responses (Regan, 1998). The subjects acted as their own controls for this experiment, with the difference between the first and last immersion being used to evaluate the effects of chronic cold exposure.

2.2.1.2 Cold-water Immersion Procedures

The immersion tank was made of stainless steel and had been previously used for underwater weighing procedures. Water was circulated by a pump (Model 413, Onga, Melbourne, Australia) at a water flow rate of $1.5 \text{ L}\cdot\text{sec}^{-1}$ and passed a water-cooling unit, consisting of a condensing unit (AH-0350-MHZ, Lovelock Luke, Australia) and a heat exchanger (CN-2, Aqua Systems Inc., Australia) and heating unit (415V Immersion Heater, Ice-Tech, Pty. Ltd., Australia). Subjects were seated on a chair in the tank with the water level reaching their fourth intercostal space. The immersion depth of the participants was dictated by the necessity to have the arms out of the water and supported by a tray across the tank. This was required to allow the blood-sampling catheter, inserted in the right arm's antecubital vein, to be held above the water on the testing days. During the cold-water immersions [$18.0^{\circ}\text{C} \pm 0.1$ (S.D.)] subjects were wearing only a swimming costume. This procedure enabled considerable heat loss without uncomfortably low skin temperatures. There were three, 60-min, cold-water stress tests (days 1, 8 and 15), and 12 adaptation immersions ($90 \text{ min}\cdot\text{d}^{-1}$). After leaving the immersion tank, subjects entered a heated room ($27\text{-}30^{\circ}\text{C}$) for rewarming.

2.2.1.3 Experimental Standardisation

To ensure that the experiments were performed with minimal influence from external factors, subjects were asked to avoid strenuous exercise and to abstain from the consumption of food, alcohol and caffeine within 12 h of each experiment. Furthermore, the experiments were performed at the same time of day to control for circadian shift in measured variables. On the test days, subjects presented at 0800 h, after an overnight fast, for blood sampling and anthropometric measurements, then consumed a standard breakfast ($38 \text{ kJ}\cdot\text{kg}^{-1}$ plus $5 \text{ ml}\cdot\text{kg}^{-1}$ fluid). Subjects were instrumented at 1100 h, rested for 30 min, and were immersed to the fourth intercostal space. Each immersion test commenced between 1200-1230 h to minimise circadian influences on baseline thermal data and plasma leptin concentration (Sinha *et al.*, 1996a). After subject had consumed the standard breakfast

2.2.1.4 Apparatus

2.2.1.4.1 Body Temperature

In the first seven subjects, T_c was recorded on test days from the oesophagus, rectum (T_{re}), and auditory canal, with only T_{re} being measured during acclimation days. Since the dynamics of T_c change were not relevant to this study, and since all indices provided qualitatively similar T_c information (Regan, 1998) only T_{re} was measured in the remaining five subjects, and herein only T_{re} is reported. Rectal temperature was recorded using a thermistor, inserted 12 cm beyond the anal sphincter (YSI type-401, Yellow Springs Instrument Co. Inc., Yellow Springs, OH, U.S.A.). These data are reported in both raw and change score formats (ΔT_{re}).

2.2.1.4.2 Skin Temperature

Skin surface temperatures (EU type, Yellow Springs Instruments Co. Inc., Yellow Springs, OH, U.S.A.) were measured at eight sites: forehead, right scapula, left upper chest, right arm, left forearm, left hand, right anterior thigh and left calf. Mean skin temperatures were derived as an area-weighted summation (ISO, 1992).

Temperatures were recorded using a data logger (1206 Series Squirrel, Grant Instruments Ltd., Cambridge, U.K.) at 15-s intervals and later downloaded to a computer for storage and subsequent analysis.

2.2.1.4.3 Subcutaneous Adipose Tissue Temperature

To evaluate the thermal impact of these trials upon subcutaneous tissue temperatures (T_{sub}), three male subjects [82.5 (± 2.2) kg; 1.83 (± 0.03) m; 24.7 (± 1.4) kg·m⁻²] were additionally exposed to a single cold-water immersion. Thermocouples housed within a teflon catheter (Physitemp Instruments Inc. Clifton, NJ, U.S.A.), were positioned in the subcutaneous adipose tissue layer of the abdomen, thigh, arm and back. These thermocouples were positioned using an 18-G hypodermic needle. This needle was inserted into a skinfold pulled away from the underlying muscle layer, and the depth of the guide needle was determined using a micrometer. The needle was then withdrawn, leaving the thermocouple at this depth until post-experimental removal. Skinfold thicknesses at the four sites were also determined to provide an estimation of the subcutaneous adipose tissue layer. On average, the thermocouples were positioned half way [50 (± 19) %] into the subcutaneous adipose tissue layer. Total and regional (leg, arm, trunk and abdomen) body fat content of these subjects was also determined using whole body Dual-Energy X-ray Absorptiometry (DEXA: XR-26, Norlad Corporation, Wisconsin, U.S.A.).

2.2.1.4.4 Cardiac Frequency

Cardiac frequency (f_c) was monitored continuously from ventricular depolarisation (Model PE3000, Polar Electro Sport Tester, Kempele, Finland), and also sampled at 15-s intervals and subsequently downloaded to a computer for storage and subsequent analysis. This system to determine f_c has previously been validated in our laboratory against a five-lead electrocardiogram (Quinton, Q500).

2.2.1.4.5 Mass, Weight and Skinfolds

Mass was determined on each test day (Model No fw-150k, AND, Milpitas, CA, U.S.A.). Skinfold thicknesses were measured at seven sites (triceps, biceps,

subscapular, supraspinale, mid-abdominal, medial calf and mid-thigh: Harpenden caliper, Eiyoken-type, Maikosha Co Ltd., Tokyo, Japan), with adiposity represented as the sum of seven skinfolds on the first test day.

2.2.1.5 Blood samples

Blood samples (10 ml from antecubital vein) were taken upon arrival (t_{baseline}), just prior to immersion (t_0), and during immersion at 25 min (t_{25}) and 60 min (t_{60}). In five subjects, a post-immersion sample (t_{120}) was obtained 60 min after commencing rewarming. Samples were kept on ice and centrifuged (2000 g) within 20 min, with separated plasma stored at -80°C for subsequent analysis. Plasma leptin concentration was determined in duplicate, using an homologous radio-immuno assay kit (SHL-81K, Linco Research, St. Charles, MO, U.S.A.).

2.2.2 Study 2: Adipose Tissue Incubation¹

The effects of reduced tissue temperature on leptin secretion were investigated *in vitro*, by comparing leptin secretion rates from human subcutaneous adipose tissue fragments incubated at three different temperatures. Tissue samples were obtained from seven female patients undergoing elective surgery [breast reduction (4) and abdominal lipectomy (3)].

Tissue samples were transported in cold saline, minced into small fragments (2-3 mm^2), and divided among three tissue culture flasks, each containing 10 ml of minimal essential medium (Hanks salts), supplemented with 10% foetal bovine serum, penicillin (100 $\text{units}\cdot\text{ml}^{-1}$) and streptomycin (100 $\mu\text{g}\cdot\text{ml}^{-1}$) per gram of tissue fragments (Gibco BRL, Life Technologies, Melbourne, Australia). After obtaining a pre-incubation medium sample, the flasks were immersed in water baths equilibrated at either 27°C , 32°C or 37°C . Samples from each of the flasks were obtained at 2, 4 and 6 h, frozen in liquid nitrogen, and stored at -80°C for subsequent leptin concentration analysis (SHL-81K, Linco Research, St. Charles, MO, U.S.A.).

¹ These experiments are described in detail in Chapter 3

2.2.3 Compartmental Model of Leptin Turnover *In Vivo*.

A compartmental model of the interaction between environmental temperature and leptin secretion was developed (Figure 2.1), based on the model of Jenkins *et al.* (2001) validated in a large sample of human twins, and used to investigate local tissue temperature influences on leptin secretion. This model permitted a mathematical evaluation of the possible impact that local tissue temperature might have upon leptin secretion *in vivo*.

The dynamics of circulating leptin, derived from this model, can be represented by:

$$dL/dt = k_1 * AT_{sub} + k_2 * AT_{visc} - k_3 * L \quad \text{Equation 1}$$

where L is the mass of leptin in the distribution volume, k_1 , k_2 and k_3 are first order, mass-specific rate constants describing the secretion of leptin from subcutaneous and visceral adipose tissue, and the clearance of leptin from the distribution volume respectively, and AT_{sub} and AT_{visc} are the masses of subcutaneous and visceral adipose tissue respectively.

These masses were estimated from skinfold thicknesses (Katch and McArdle, 1973) assuming a subcutaneous:visceral mass ratio of 60:40 (Seidell *et al.*, 1990). At steady state ($dL/dt = 0$), equation (1) reduces to:

$$L = [k_1 * AT_{sub} + k_2 * AT_{visc}] / k_3 \quad \text{Equation 2}$$

If it is assumed that the effects of temperature on k_3 are insignificant, the proportional change in leptin mass resulting from an isolated change in temperature in subcutaneous adipose tissue can then be obtained from:

$$L(T_2)/L(T_1) = [k_1(T_2) * AT_{sub} + k_2 * AT_{visc}] / [k_1(T_1) * AT_{sub} + k_2 * AT_{visc}] \quad \text{Equation 3}$$

where T_1 and T_2 are two arbitrary temperatures, $L(T_i)$ represents leptin mass as a function of temperature, and $k_1(T_i)$ represents k_1 as a function of temperature.

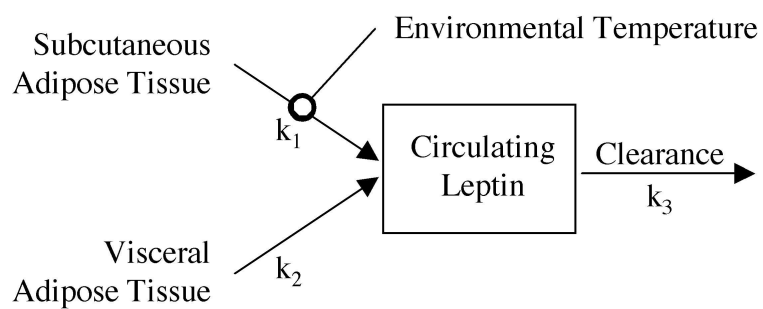


Figure 2.1 Hypothetical model of local temperature effects on plasma leptin concentration. Leptin is secreted from subcutaneous and visceral depots into the circulation, according to rate constants k_1 and k_2 , respectively, and is cleared predominantly by the kidney (k_3). During cold exposure, leptin secretion from the subcutaneous depots is affected by changes in local temperature, while leptin secretion from visceral depots remains stable.

An estimate of the effect of temperature on leptin secretion from adipose tissue [$k_1(T)$] was obtained from log-linear regression analysis of the results of the *in vitro* studies:

$$k_1(T) = dL/dt = \text{Exp} (b*[T-c]) \quad \text{Equation 4}$$

where b and c are respectively the slope and X -intercept of the linear regression relationship between Ln-transformed adipose tissue mass-specific leptin secretion rate and incubation temperature (T).

The effects of cold-water immersion on circulating leptin *in vivo* were predicted from the model by substituting the *in vitro* derived estimate of $k_1(T)$ into equation (3) with T_1 and T_2 set to the pre-immersion and immersion values respectively for the measured T_{sub} . This use of the model depends on the validity of the following assumptions: (1) the conditions *in vivo* at the end of cold-water immersion are sufficiently close to a steady state; (2) the effects of cold-water immersion on k_3 are insignificant compared to the temperature effects on k_1 ; (3) k_2 is not significantly affected by cold-water immersion; (4) adipose tissue from females incubated *in vitro*, displays the same proportional effects of temperature on leptin secretion as does subcutaneous adipose tissue in males *in vivo*; (5) cold immersion-induced changes in plasma volume are insignificant compared to changes in the plasma leptin mass, and thus changes in leptin mass closely approximate changes in circulating leptin concentration; (6) the total adipose tissue mass is distributed between subcutaneous and visceral depots in the ratio of 60:40; and (7) the subcutaneous adipocyte leptin secretion rate has been estimated to be twice that of visceral adipocytes (Van Harmelen *et al.*, 1998; Gottschling-Zeller *et al.*, 1999).

2.2.4 Design and Analyses

The immersion study was based on a repeated-measures design, with data analysed at four time points (baseline, 0, 25, 60 min) on each of days 1, 8 and 15. In five subjects only, an additional sample was taken after 60 min of rewarming. The *in vitro* study was also based on a repeated-measures design, with four time points (baseline, 2, 4, 6 h) for each of the three incubation temperatures. Data were analysed

using either two-way analysis of variance or paired *t*-tests. Simple and partial correlation analyses were performed between T_{re} and log-transformed plasma leptin concentration $\text{Log}_e(\text{leptin})$, since leptin data are log-normally distributed. An hypothesised relationship between plasma leptin concentration and the acute T_{re} response to cold immersion was tested by modelling the covariances between $\text{Log}_e(\text{leptin})$ and ΔT_{re} across all three trials, using structural equation modelling software (Neale, 1997). This analysis provided maximum likelihood fits to covariance matrices by models incorporating the within subjects (trial) covariances of $\text{Log}_e(\text{leptin})$ and of ΔT_{re} , with or without a trial-independent covariance between $\text{Log}_e(\text{leptin})$ and ΔT_{re} . Goodness of fit was assessed using a χ^2 test. The results are presented as a standardised path coefficient (correlation coefficient) with 95% confidence intervals (CI). The χ^2 of a model incorporating the relationship between $\text{Log}_e(\text{leptin})$ and T_{re} was compared to the χ^2 of a reduced model without this relationship (Neale *et al.*, 1994). Alpha was set at the 0.05 level for all analyses. Data for all dependent variables are presented as means with standard errors of the means, unless otherwise indicated.

2.3 RESULTS

2.3.1 Study 1: Cold-water Immersion:

2.3.1.1 Thermal and Cardiac Responses to Cold-Water Immersion

Pre-immersion T_{re} , T_{sk} and f_c averaged $37.3 (\pm 0.1) ^\circ\text{C}$, $27.7 (\pm 0.2) ^\circ\text{C}$ and $82 (\pm 7) \text{ b}\cdot\text{min}^{-1}$, respectively (Figure 2.2). Cold-water immersion elicited a rapid T_{sk} reduction, with no further change between 25-60 min (Figure 2.2). Subcutaneous adipose tissue temperatures (T_{sub}) decreased rapidly during immersion. The average temperature of the three immersed depots fell from 32.9°C to 23.3°C within the first 10 min, then stabilised at 21.8°C after 30 min. In contrast, the subcutaneous temperature of the non-immersed arm slowly decreased from 32.6°C to 29.9°C . Using the assumption that 60% of total body fat is stored subcutaneously (Seidell *et al.*, 1990), and DEXA scans which indicated that 11% of the total subcutaneous adipose tissue was located in non-immersed regions for our subjects, it was estimated that the overall decrease in T_{sub} was approximately 10.2°C .

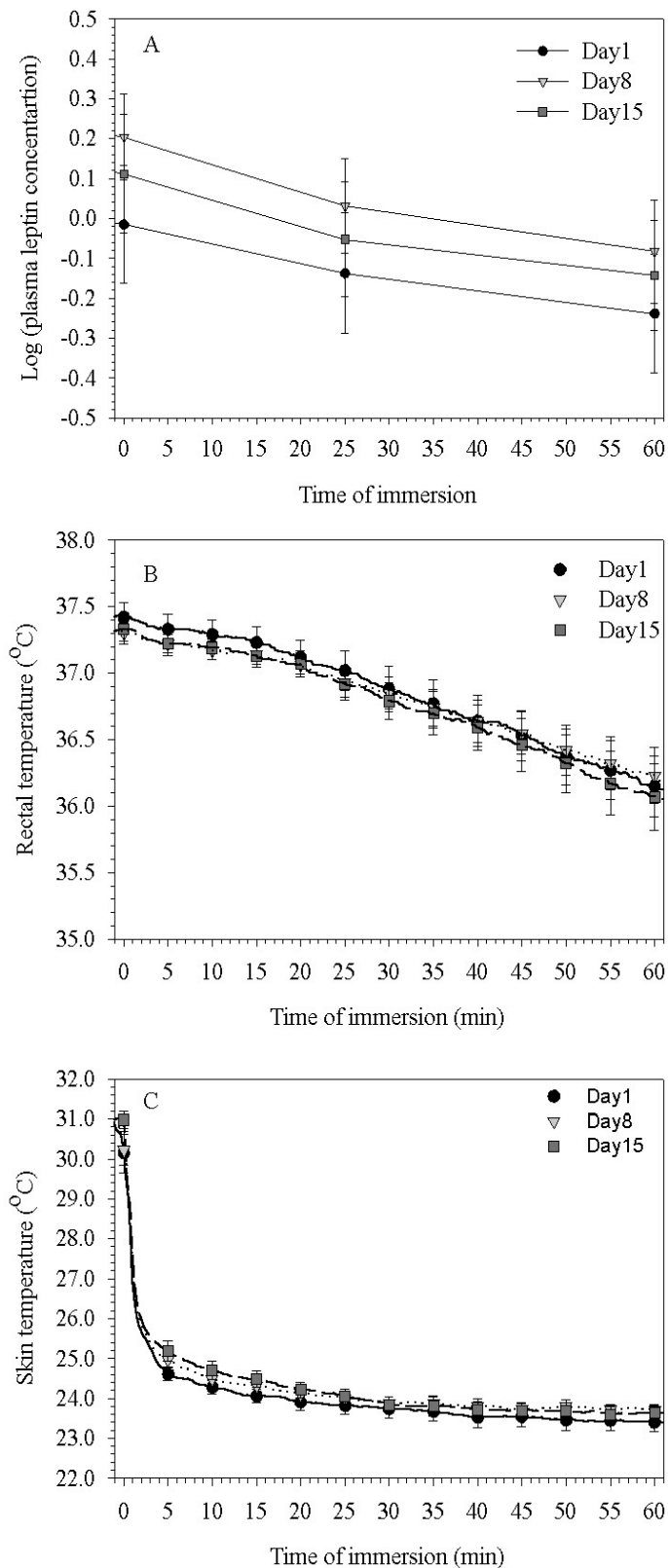


Figure 2.2: Log-transformed plasma leptin concentration (A), rectal (B) and mean skin temperatures (C) during cold-water immersion (18°C), on three test days (day 1, day 8, and day 15) during a 2-week cold-water adaptation experiment. Data are means (S.E.M.).

Following an initial delay, T_{re} progressively declined ($P<0.05$; Figure 2.2). Cardiac frequency displayed the typical, cold-induced bradycardia, being significantly lower than t_0 ($P<0.05$), and averaging $72 (\pm 3) \text{ b}\cdot\text{min}^{-1}$. No significant adaptation trends were apparent within the thermal or f_c data over the course of the 15-day experiment.

2.3.1.2 Effect of Cold-Water Immersion on Plasma Leptin Concentration

Fasting plasma leptin concentration [$1.5 (\pm 0.1) \text{ ng}\cdot\text{ml}^{-1}$] was significantly higher than immediately prior to immersion [$1.2 (\pm 0.1) \text{ ng}\cdot\text{ml}^{-1}$; $P<0.05$], with this difference ($\sim 6\%\cdot\text{h}^{-1}$) being consistent with the reported diurnal change in leptin secretion (Sinha *et al.*, 1996a). Plasma leptin concentrations, during repeated cold-water immersions, revealed two significant response patterns (Figure 2.2).

First, there was an acute effect, apparent within each immersion, and resulting in significant plasma leptin concentration reductions at both t_{25} (-14%) and t_{60} (-22%; $P<0.05$). In five subjects, from whom blood samples were collected following rewarming, this acute effect on leptin concentration was substantially reversed after 60 min of rewarming (t_{120}), and was significantly greater than at t_{60} [$1.1 (\pm 0.2)$ versus $0.9 (\pm 0.2) \text{ ng}\cdot\text{ml}^{-1}$, respectively; $P<0.05$].

Second, a chronic, possible adaptation effect was evident, which developed over the 15 days. The plasma leptin concentrations for days 8 and 15 were elevated relative to that observed on day 1 ($P<0.05$, Figure 2.2). This return towards the normal circulating concentration was apparently unrelated to adipose mass, as total mass remained stable [day 1: $72.1 (\pm 2.4) \text{ kg}$; day 8: $72.1 (\pm 2.4) \text{ kg}$; day 15: $72.3 (\pm 2.5) \text{ kg}$; $P>0.05$]. Although no significant trial effect was apparent on the ΔT_{re} , the pattern of the ΔT_{re} was similar to that of the plasma leptin concentration [day 1: $-1.4 (\pm 0.2) ^\circ\text{C}$; day 8: $-1.2 (\pm 0.2) ^\circ\text{C}$; day 15: $-1.3 (\pm 0.2) ^\circ\text{C}$]. Analyses of covariance, within and between plasma leptin concentrations and ΔT_{re} data, across the three test days, revealed a relationship between $\text{Log}_e(\text{leptin})$ and ΔT_{re} , such that higher leptin concentrations prior to immersion were associated with smaller ΔT_{re} during immersion (standardised path coefficient 0.45, 95% CI 0.04-0.84). Omitting this relationship from the model significantly degraded the fit [$\chi^2 = 17.5$ (16 *df*, $P>0.05$) versus 22.2 (17 *df*, $P>0.05$), difference 4.3 (1 *df*, $P<0.05$)].

2.3.2 Study 2: Adipose Tissue Incubation:

2.3.2.1 Incubation Temperature Effects on Leptin Secretion

Leptin secretion rates for adipose tissue fragments, incubated at the three different temperatures, are shown in Figure 2.3. A reduction of the incubation temperature significantly decreased leptin secretion rate ($P < 0.05$), such that tissue fragments incubated at 27°C had a secretion rate of $1.2 \text{ ng} \cdot \text{h}^{-1} \cdot \text{g}^{-1}$ of tissue, compared with $4.4 \text{ ng} \cdot \text{h}^{-1} \cdot \text{g}^{-1}$ when incubated at 37°C.

Using the derived model (Figure 2.1), the local effect of reduced subcutaneous adipose tissue temperature on leptin secretion rate was predicted to decrease circulating leptin concentration from 29% to 41%, with the assumed ratio of k_1 and k_2 ranging from 1:1 to 2:1, respectively. Thus, the predicted reduction in leptin secretion rate, due to direct local thermal effects, was greater than the observed 22% decrease in circulating leptin in the cold-water immersion experiments.

2.4 DISCUSSION

2.4.1 Cold Exposure Effects on Circulating Leptin

Four significant observations were derived from these two experiments. First, acute cold-induced negative effects upon plasma leptin concentration in humans were demonstrated to occur during cold-water immersion. This observation supported our primary working hypothesis. Second, although a centrally-mediated neural component cannot be excluded, it was shown that the local direct effects of reduced T_{sub} on leptin secretion rate were strong enough to completely account for the observed *in vivo* decrease in plasma leptin concentration. Third, a unique and significant relationship between plasma leptin concentration and the fall in T_{re} during cold-water immersion was found. Finally, it was demonstrated for the first time, a longer-term, positive effect of repeated cold exposure on plasma leptin concentration. As with many hormones, plasma leptin concentration shows a diurnal rhythm (Sinha *et al.*, 1996a), peaking around midnight, and dropping during the morning to reach its lowest concentration around 1200 h. In this study, and consistent with such a diurnal pattern, leptin

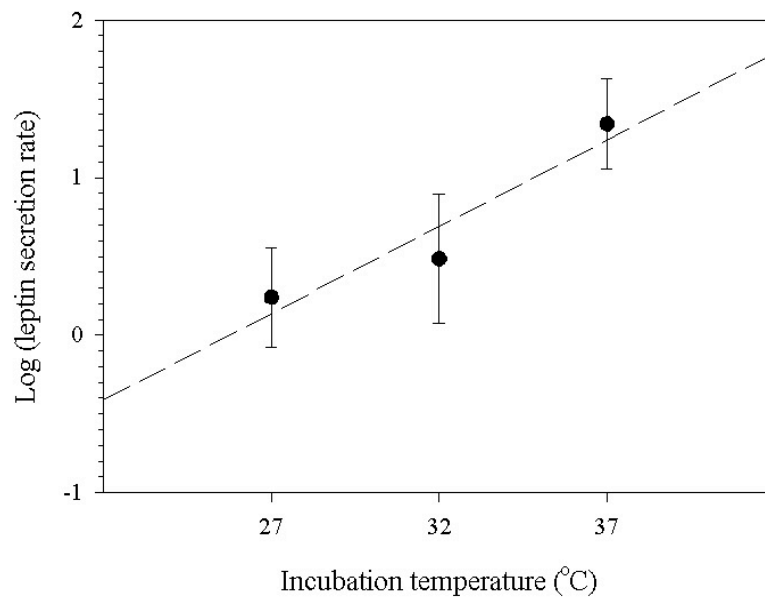


Figure 2.3: In vitro leptin secretion rates of human subcutaneous adipose tissue fragments, incubated at three temperatures (27, 32 and 37°C). Fragments were obtained from woman (n=7) during abdominal lipectomy and breast reduction procedures. Data are means (S.E.M.).

concentration decreased at a rate of approximately $6\% \cdot h^{-1}$ between the baseline and pre-immersion samples (t_0), comparable to the $9\% \cdot h^{-1}$ reduction observed by Sinha *et al.* (1996) over a similar period. However, during the first 25 min of cold-water immersion (1200-1300 h), circulating leptin concentration decreased at a rate of $34\% \cdot h^{-1}$, at which time (Sinha *et al.*, 1996a), reported a stable concentration. Since this reduction is far in excess of that which may reasonably be expected for diurnal variation, it can be concluded that the observed plasma leptin concentration reduction represents an acute response to cold stress, and cannot be ascribed to the diurnal pattern of leptin secretion.

2.4.2 Possible Mechanisms of Cold-Induced Changes in Circulating Leptin

Four possible mechanisms could account for this acute decrease in plasma leptin concentration. These relate to reduced leptin secretion, localised subcutaneous pooling of secreted leptin due to altered perfusion of subcutaneous adipose tissues, an increased leptin clearance during cold exposure, or an increase in diluting (plasma) volume into which the leptin is eventually secreted.

First, one may hypothesise a cold-induced suppression of either leptin production or secretion, either locally- or centrally mediated, with the latter occurring via the sympathetic nervous system. Peino *et al.* (2000) reported reductions in leptin secretion rates from human omental adipose tissue incubated at lower temperatures. However, since similar changes were observed for growth hormone secretion, the authors concluded these reductions were non-specific, and had little physiological relevance. This conclusion can be disputed. Furthermore, while not disputing the functional capacity of the sympathetic nervous system to change leptin secretion, our data support the case that local thermal effects can powerfully modulate its secretion. The possible coexistence of centrally- and peripherally-mediated control has considerable cybernetic appeal, since, like body temperature regulation, the latter provides the means through which rapid, transient and subtle changes in environmental temperature may modify physiological function.

Using our *in vitro* data to model local effects of a reduced T_{sub} on leptin secretion, the predicted decrease in secretion, due to these local effects, was almost twice that observed during acute cold-water immersion. Assuming that the latter is more

indicative of secretion than removal, then these results indicate that the local responses to changes in adipose tissue temperature can significantly determine plasma leptin concentration, even in the absence of sympathetic outflow, and may therefore potentially modify energy balance *in vivo*.

The cold-induced suppression of leptin synthesis and secretion is supported by previous animal data (Moinat *et al.*, 1995; Trayhurn *et al.*, 1995; Hardie *et al.*, 1996b; Bing *et al.*, 1998; Evans *et al.*, 1999). The contribution of these direct thermal effects to adipocyte function is unclear, but would presumably be dependent upon the extent to which tissue temperature was modified. The derived model predicts a 22% fall in plasma leptin concentration with the local temperature effects of a 2°C decrease in body temperature. This result is slightly less than the 30% decrease reported in mice under similar conditions (Evans *et al.*, 1999). Thus, this 2°C decrease probably underestimates actual changes in visceral and subcutaneous temperatures. Therefore, it is suggested that cold-induced decreases in circulating leptin can, at least partially, be explained by local temperature effects.

Others have concluded that cold-induced sympathetic activation plays the primary role in the cold-induced suppression of circulating leptin in mice (Trayhurn *et al.*, 1995; Deng *et al.*, 1997; Evans *et al.*, 1999). However, agonist studies demonstrate only the potential effects of sympathetic activation, rather than its specific involvement. Evans *et al.* (1999) investigated beta-adrenergic blockade on cold-induced modifications of serum leptin concentration and *ob* mRNA expression in brown and white adipose tissue in mice. As circulating leptin and *ob* gene expression were not significantly reduced in all the experiments with beta-adrenergic antagonist, the authors concluded that blockade prevented cold-induced changes. This interpretation is open to opposition, as it is based upon the assumption that a failure to detect an effect was equivalent to demonstrating that no effect existed. We suggest that if more appropriate analyses had been undertaken (i.e. factorial *ANOVA*), their data may well have shown that the blockade did not modify thermally-induced changes.

Second, it may be argued that changes in circulating leptin concentration result simply from decreased blood flow to, and from, subcutaneous adipose tissue, and consequently reduced leptin release into the circulation. Indeed, the rewarming period

was designed to help evaluate this possibility. Our *in vivo* data, in the absence of skin blood flow measures, showed that plasma leptin concentration returned towards baseline, and significantly exceeded levels at the end of the cold exposures. Unfortunately, this observation does not allow one to differentiate between blood restoration and the removal of a locally-mediated cold suppression. While this work was in progress (Zeyl *et al.*, 1998), Ricci *et al.* (2000) demonstrated a similar 22% decrease in plasma leptin concentration during cold exposure, while simultaneously observing a doubling of plasma glycerol concentration. As it was unlikely that such an increase in lipolysis could be wholly attributable the visceral depot in their lean, healthy women, they concluded that blood flow remained sufficient for drainage of adipose products during cold exposure. Regardless of changes in tissue perfusion, our *in vitro* data, obtained from isolated tissue samples, can more than account for the changes observed in plasma leptin concentration *in vivo*. That is, the thermal effects on leptin secretion *in vitro* exceeded those observed *in vivo*. Thus, while changes in tissue perfusion could account for the current observations, they can also be wholly explained on the basis of local thermal affects on subcutaneous adipocytes.

Third, altered leptin clearance can affect the circulating leptin concentration independently of local thermal or blood flow influences. The kidneys account for 50-80% of the plasma leptin clearance, being dependent on renal plasma flow rather than glomerular filtration rate (Meyer *et al.*, 1997; Garibotto *et al.*, 1998). However, the effects of cold-water immersion on renal haemodynamics in humans are unclear. While renal plasma flow is significantly reduced [-11.4% (\pm 13.6 S.D.)] during cold-air exposure in 15°C (Atterhog *et al.*, 1975), there appear to be no data on renal haemodynamics for cold-immersed humans. The effects of thermoneutral immersion on renal haemodynamics are inconsistent (Epstein, 1976; Coruzzi *et al.*, 1986). Therefore, at present, the possibility that a cold-induced change in leptin clearance underlying changes in plasma leptin concentration under our conditions can neither be supported nor be eliminated.

Fourth, a change in plasma volume may modify the concentration of any circulating element. Data from our laboratory (Regan, 1998) and others (Atterhog *et al.*, 1975; Sramek *et al.*, 1993) demonstrate that cold exposure decreases rather than increases plasma volume. Therefore, changes in plasma volume are not likely to

contribute to the observed decrease in plasma leptin concentration. However, cold-induced decreases in plasma volume, and possibly leptin clearance, may counter balance the decrease in plasma leptin concentration *in vivo*, resulting in an overestimation of the response by the above model.

2.4.3 Effect of Repeated Cold Exposure on Plasma Leptin Concentration

In addition to the above acute effects, this study has uniquely demonstrated an apparent adaptation effect on plasma leptin concentration. Repeated cold exposure allowed for a significant restoration of the circulating leptin concentration. This change could not be accounted for by changes in body mass over this period. Instead, the weak correlation between plasma leptin concentration and ΔT_{re} may be indicative of a protective response to reduce the fall in T_{re} during cold exposure. This was suggested by Stehling *et al.* (1997) and indirectly supported by the observations of Martinez *et al.* (1998). At the very least, the present results are consistent with a role for leptin in the thermoregulatory responses to cold.

2.4.4 Conclusions

In summary, this study replicated, in parallel studies, previous work by others in humans, which have separately demonstrated cold-induced reductions in circulating leptin concentration *in vivo* (Ricci *et al.*, 2000) and leptin secretion *in vitro* (Peino *et al.*, 2000). However, in contrast to the conclusions drawn from those studies, when our data are compared using a model of temperature effects on leptin concentration *in vivo*, they support an important contribution of local adipose tissue temperature to circulating leptin concentration. Furthermore, this study is the first to show separate and opposing effects of acute and repeated cold exposure on plasma leptin concentration in humans. The present data are supportive of a role for leptin in both acute and longer-term thermoregulatory responses to cold exposure. This study has demonstrated that local effects of a reduced T_{sub} may substantially contribute to the acute decrease in plasma leptin concentration during immersion. It is therefore our view that, while the sympathetic nervous system can regulate leptin secretion, locally-mediated modulation is also possible, and even substantial, having been shown herein to occur in the absence of the former. Indeed, it is believed both avenues coexist, as they do for temperature

regulation. Since leptin is involved in the regulation of energy intake and expenditure, these results may identify potential mechanisms linking environmental temperature and behavioural responses to the regulation of energy balance and body composition.

CHAPTER 3: EFFECTS OF INCUBATION TEMPERATURE ON ADIPOSE TISSUE METABOLISM AND LEPTIN SECRETION RATE

3.1 INTRODUCTION

Temperature effects on plasma leptin concentration and *ob* gene expression have been shown in human, rodent and sheep experiments (Trayhurn *et al.*, 1995; Hardie *et al.*, 1996b; Puerta *et al.*, 2002; Asakuma *et al.*, 2003). However, the underlying mechanism for the inhibitory effects of cold exposure on leptin physiology has not yet been fully identified. Cold-induced activation of the sympathetic system, resulting in stimulation of beta-adrenoreceptors in adipose tissue, is often considered as the underlying mechanism for the observed decreases in circulating leptin and *ob* gene expression during cold exposure (Trayhurn *et al.*, 1995; Trayhurn *et al.*, 1999; Rayner and Trayhurn, 2001). Local, direct effects of reduced temperature of adipose tissue itself are rarely considered as a contributing factor. The direct temperature effects on leptin physiology have been previously investigated using human omental adipose tissue (Peino *et al.*, 2000) and rat adipocytes (Bradley *et al.*, 2001). Both studies demonstrate that leptin secretion reduces when the incubation temperature is decreased. Peino *et al.* demonstrated significantly lower leptin secretion from human omental adipose tissue incubated at 32°C compared to 37°C. Since similar results were observed in GH secretion from GH3 cell, it was suggested by the authors that these observations have no physiological implications. Furthermore the physiological significance of this study can be questioned, as omental adipose tissue is not likely to be exposed to temperatures as low as 32°C.

The temperature of subcutaneous adipose tissue (T_{sub}) in humans varies over even wider temperature ranges than applied in the study of Peino *et al* (2000). Variations in T_{sub} have been demonstrated both between and within different subcutaneous adipose tissue depots. For example, in a resting subject, T_{sub} varies by 2-3°C at an ambient temperature of 27°C. However, when the environmental temperature decreased to 23°C, the difference between depots increased to 5°C (Webb, 1992). Data from cold-water immersion experiments, discussed in the previous chapter, show that variations within one site can be as large as 10°C during cold-water immersion.

Changes in T_{sub} occur rapidly during cold-water immersion and reach equilibrium with a water temperature of 18°C within 25 minutes.

It was therefore of interest to investigate if a similar direct effect of temperature on leptin secretion rate could be demonstrated in subcutaneous adipose tissue and whether the magnitude of such effect could be quantified and qualified as important contributor to the *in vivo* response of cold-exposure on circulating leptin

3.1.1 Adipose Tissue: *In Vitro* Experiments.

Investigations using *in vitro* culture techniques provide a controlled environment, in which the direct temperature effects on leptin physiology and adipose tissue metabolism can be studied. Also in these type of experiments, metabolic investigations can be combined with investigations studying the effects on gene transcripts and products using the same tissue preparation (Arner, 1995). Studies into the regulation of leptin secretion commonly use freshly isolated adipocytes or adipose tissue fragments in primary culture. Leptin secretion in human adipose tissue has been shown to be an exclusive function of the adipocytes itself, rather than also of other cells that are present in the tissue (Fain *et al.*, 2002). Studies using adipose tissue fragments have several advantages over isolated adipocytes since the tissue remains relatively intact. Thus there is no selective loss of small adipocytes. Neither are there confounding effects of the proteolytic enzyme collagenase used to breakdown the connective tissue. Additionally, there will be no inhibitory effect on lipolysis by leaked adenosine from isolated adipocytes. One disadvantage of using tissue fragment is that the responses to hormones and other active agents in the culture medium may be slower than in isolated adipocytes due to the diffusion barriers present in the tissue.

3.1.1.1 Adipose Tissue Sampling

There are several methods to obtain human adipose tissue sample for culture studies. Commonly reported methods in studies using human adipose tissue are: 1) peri-operative tissue sampling, 2) needle biopsy aspiration and 3) manual liposuction. The advantages and disadvantages of these three methods are summarised in Table 3.1.

Table 3.1: Advantages and disadvantages of three commonly used adipose tissue sampling procedures.

Method	Advantage	Disadvantage
Per-operative adipose tissue sampling	<ul style="list-style-type: none"> • No extra inconvenience for the subject. • Substantial amount of tissue can be easily obtained. 	<ul style="list-style-type: none"> • Requires involvement and co-operation of medical staff. • Immediate transport required to laboratory after removal • Use of anaesthetic reagent
Needle biopsy method	<ul style="list-style-type: none"> • Easy to apply within a research laboratory. • Rapidly performed 	<ul style="list-style-type: none"> • Small quantities (<34 mg) • Suction can disrupt the adipocytes
Manual liposuction	<ul style="list-style-type: none"> • Reasonable amount of tissue that can be obtained (1-5 gr). 	<ul style="list-style-type: none"> • Invasive method (approx 5mm incision in skin required). • Use of anaesthetic reagent

Both preoperative tissue sampling and liposuction provide reasonable quantities of tissues needed for experiments that involve multiple treatments and conditions. These methods however do require the use of an anaesthetic reagent, which may have confounding effects on adipose tissue metabolism. However, studies to the effects of anaesthetics demonstrate that the effects of local and general anaesthetic on the metabolic processes of isolated adipocytes are limited (Arner *et al.*, 1973; Kolaczynski *et al.*, 1994). For example, lidocaine and propofol do not alter basal lipolysis (Arner *et al.*, 1973), nor the adrenergic regulation of lipolysis (Large *et al.*, 1997)

3.1.2 Regulation of Leptin Physiology

3.1.2.1 Regulation of Leptin Physiology - *In Vivo* Studies

Involvement of the adrenergic sympathetic system in the regulation of leptin is supported by studies using epinephrine and the beta-adrenergic agonist, isoprenaline. Epinephrine infusion significantly decreased leptin concentrations both in lean and obese women (Couillard *et al.*, 2002). Circulating leptin concentration was rapidly decreased by isoprenaline, with maximal suppression [20.5 (15.0-25.0)]% of pre-infusion concentration, observed after 2 hr. In the recovery period of 1 hr, plasma leptin concentrations rapidly returned to pre-infusion levels (Pinkney *et al.*, 1998).

Besides the beta-adrenergic pathways, also insulin, glucocorticoids have been suggested to be involved in the regulation of leptin physiology. Although hyperinsulinemia did not show any acute effects of insulin on circulating leptin levels in both lean and obese subjects (Caprio *et al.*, 1996; Kolaczynski *et al.*, 1996), prolonged hyperinsulinemia (up to 72 hrs) resulted in increased plasma leptin concentration (Kolaczynski *et al.*, 1996). The effects of dexamethasone on circulating leptin levels in healthy male subjects have also been investigated. One single oral dose of dexamethasone increased plasma leptin concentration, reaching peak concentration 15 h after ingestion. Repeated dexamethasone administration, twice a day for three subsequent days, produced a sustained elevation in plasma leptin concentration reaching the highest concentration at the second day (Miell *et al.*, 1996).

3.1.3 Regulation of Leptin Physiology - *In Vitro* Studies

While *in vivo* studies suggest involvement of insulin, glucocorticoids and the beta-adrenergic pathway in leptin regulation, the results from various rodent, ruminant and human adipose tissue studies are not consistent. Some studies demonstrate stimulatory effects of insulin alone or in combination with dexamethasone, a synthetic glucocorticoid, on leptin secretion and *ob* gene expression (Hardie *et al.*, 1996a; Kolaczynski *et al.*, 1996; Russell *et al.*, 2001; Faulconnier *et al.*). In contrast others found that insulin prevented dexamethasone induced increases in either *ob* mRNA expression or leptin release (Considine *et al.*, 1997; Halleux *et al.*, 1998a). These studies with conflicting results differ with respect to the duration of the incubation period, culture medium, the hormonal concentrations.

Results of Ricci *et al.* demonstrate also differences in the regulation of leptin physiology between species. Studies using human adipose tissue of obese and non-obese subjects showed no acute effect (<3 h) of insulin on leptin secretion, while isoproterenol significantly reduced leptin secretion. In contrast, in rat adipose tissue insulin significantly increased the total amount of leptin secreted, while isoproterenol alone or in combination with insulin did not affect leptin secretion compared to the control condition (Ricci *et al.*, 2005).

Depot-specific differences with respect to leptin secretion and *ob* mRNA expression are unambiguously documented (Hube *et al.*, 1996; Montague *et al.*, 1997; Van Harmelen *et al.*, 1998). Human studies demonstrate that the expression of *ob* mRNA is higher in subcutaneous adipose tissue than omental tissue. The subcutaneous to omental ratio ranges from 1.9 in men up to 5.5 in women (Montague *et al.*, 1997). While leptin secretion rate from subcutaneous adipose tissue is two to three times higher than from omental tissue (Van Harmelen *et al.*, 1998). Furthermore, differences in the regulation of leptin physiology between depots have also been demonstrated (Russell *et al.*, 2001). While dexamethasone increased *ob* mRNA expression in both omental and subcutaneous adipose tissue, leptin secretion was only increased in omental tissue by dexamethasone. In contrast, insulin did not affect *ob* mRNA expression in either depots but increased leptin secretion from the subcutaneous adipose tissue fragments.

The main findings of the regulation of leptin physiology of *in vitro* studies using human adipose tissue are summarized in Table 3.2. Whether or not the hormonal regulation of leptin physiology is affected by temperature has to our best knowledge not yet been determined.

3.1.4 Summary

Both plasma leptin concentration and *ob* gene expression have been shown to decrease during cold exposure in (Trayhurn *et al.*, 1995 ; Hardie *et al.*, 1996b; Ricci *et al.*, 2000; Puerta *et al.*, 2002; Asakuma *et al.*, 2003). Increased sympathetic tone has often been considered as the underlying mechanism (Trayhurn *et al.*, 1995; Trayhurn *et al.*, 1999; Rayner and Trayhurn, 2001). However, the possibility of local contributing effects has hardly been investigated. Direct, local temperature effects on leptin secretion have been previously demonstrated (Peino *et al.*, 2000) in human omental adipose tissue but not in subcutaneous adipose tissue, a depot that is subjected to large variations in temperature during cold-exposure.

Tissue culture using human subcutaneous adipose tissue fragments is a well-established and reliable method, which provides a controlled environment to determine local temperature effects on leptin secretion rate.

Insulin, dexamethasone and the beta-adrenergic receptors are involved in the regulation of leptin production. To the best of our knowledge no previous investigation has determined whether or not there is any interaction between the regulation of leptin physiology and temperature.

The present study sought to quantify local temperature effects on leptin physiology, and to determine if the regulation of leptin by insulin, dexamethasone and isoprenaline is affected by incubation temperature using human subcutaneous adipose tissue explants. Subcutaneous adipose tissue was obtained from patients undergoing surgery. Fragments of adipose tissue were incubated at different temperatures (27°, 32°C

Table 3.2: Summary of results of *in vitro* experiments to hormonal regulation of leptin secretion using human adipose tissue ('INS'=insulin, 'DEX'=dexamethasone, 'ISO'=isoprenaline, '+' indicates stimulatory and '-' indicates negative effect on leptin secretion).

and 37°C) in the absence and presence of insulin and dexamethasone, isoprenaline or the combination of all three.

3.2 METHODS

Subcutaneous adipose tissue samples were obtained from seven female patients undergoing elective surgery [breast reduction (4) and abdominal lipectomy (3)]. Tissue samples were transported in cold saline to the laboratory. On arrival, the tissue was immediately minced into small fragments (2-3 mm³) with sharp sterilized scissors. Tissue fragments were divided between three tissue culture flasks, each containing 10 ml of culture medium per gram of tissue fragments (Minimal Essential Medium (Hanks' salts), supplemented with 10% foetal bovine serum, Penicillin (100 units/ml) and Streptomycin (100 µg/ml), all Gibco BRL, Life Technologies, Melbourne, Australia). To study the effects of insulin and dexamethasone and/or isoprenaline, the culture medium was supplemented with insulin (7 nM \approx 1.17 mIU/ml) and dexamethasone (25 nM) and/or isoprenaline. (10 µM). After obtaining a pre-incubation medium sample, the flasks were immersed in three different water baths set at 27, 32 and 37°C respectively. At 0, 2, 4, 6, 10 and 22 h of incubation, samples of the culture medium (1.5 ml) were collected. Medium samples were frozen in liquid nitrogen and stored at -80°C. These were later analysed for leptin, non-esterified fatty acids (NEFA) and glucose concentration.

3.2.1 Leptin

Leptin secretion into the culture medium was determined using a human leptin specific RIA (Linco, St Charles, MO, U.S.A.). As recommended by the manufacturer, the quantification was performed in duplicate using 100 µg of culture medium for each determination. The limit of sensitivity was 0.5 µg/l, the intra-assay coefficient of variation was 5.2% and the inter-assay coefficient of variation was 4.9%. Leptin secretion is expressed as rate in ng·hr⁻¹ per g wet weight of tissue (ng·hr⁻¹·g⁻¹) or as total amount of leptin secreted at a certain time point in ng·g⁻¹.

3.2.2 Non-Esterified Fatty Acids

The concentration of non-esterified free fatty acids (NEFA) in the culture medium was measured using an enzymatic, colorimetric method (NEFA C, Wako Pure Chemical Industries Ltd., Osaka, Japan). The aliquots of culture medium were processed on a COBAS Mira Plus system (Roche Diagnostics Pty. Ltd., Castle Hill, Australia). NEFA release in the culture medium is expressed as $\text{nMol}\cdot\text{gr}^{-1}$ of adipose tissue.

3.2.3 Glucose

Glucose concentration of the culture medium was determined using an enzymatic, colorimetric methods (Glucose HK, Roche Diagnostics Pty. Ltd., Castle Hill, Australia). Samples were processed on a COBAS Mira Plus system (Roche Diagnostics Pty. Ltd., Castle Hill, Australia). Glucose uptake was determined by the calculation of differences in glucose concentration of the culture medium at various points in time (Halleux *et al.*, 1998a; Faulconnier *et al.*, 2003). Glucose uptake is expressed as $\mu\text{mol}\cdot\text{gr}^{-1}$ of adipose tissue.

3.2.4 Design

This *in vitro* study was based on a repeated-measures design, with one within factor (time) and two between factors (incubation temperature and treatment). Data were analysed by Analysis of Variance (*ANOVA*) and Multivariate Analysis of Variance (*MANOVA*).

Data analysis of leptin secretion, glucose uptake and NEFA release demonstrated non-linear relationships over time. Moreover, the data seems to indicate that decay processes within the adipose tissue fragments occurred during the 22 h of incubation. Therefore only data of the first six hours of incubation were included in the analysis.

3.3 RESULTS

Total amounts of leptin and NEFA released at the five time-points during the incubation period are presented in Figures 3.1 A and B. The data represents the means of the three different incubation temperatures (27, 32 and 37°C). As discussed in the method section, the time course data suggest that a certain level of decay of adipose tissue fragments may have occurred during the second half of the incubation. For example, when expressed as an hourly rate, leptin secretion rate decreased from 4.3 (± 0.5) ng·hr⁻¹·gr⁻¹ of tissue in the first 2 h to 1.7 (± 0.5) ng·hr⁻¹·gr⁻¹ in the last 12 h of the 22 h of incubation ($P < 0.05$). While isoprenaline showed to be a very potent stimulus of NEFA release, after 10 h of incubation the rate of NEFA release was almost reduced to zero in all conditions. Therefore, data of only the first six hours of the incubation period were included in the analysis.

3.3.1 Temperature effects on leptin secretion rate in the control condition

There was no significant difference in leptin secretion rate or total amount secreted between the two subcutaneous adipose tissue depots (breast versus abdominal, $P > 0.05$), therefore all data was pooled together in subsequent analyses. The means of leptin secretion rates during the first 6 hours of incubation in control conditions at the three incubation temperatures are shown in Figure 3.2. At the lower incubation temperatures, leptin secretion rate was significantly decreased ($P < 0.05$). In adipose tissue fragments incubated at 27°C leptin secretion rate was 1.9 (± 0.4) ng·hr⁻¹·gr⁻¹ of tissue, compared with a rate of 4.8 (± 0.4) ng·hr⁻¹·gr⁻¹ of tissue when incubated at 37°C.

3.3.2 Temperature and Hormonal Effects on Leptin Secretion

When data of all four incubation conditions are incorporated in the analysis, the effect incubation temperature remains evident ($P < 0.05$). Figure 3.3 shows the means of hourly leptin secretion rate during the first six hours at three incubation temperatures for the four medium conditions. The supplementations of the culture medium did not affect leptin secretion in any of the conditions (treatment $P > 0.05$).

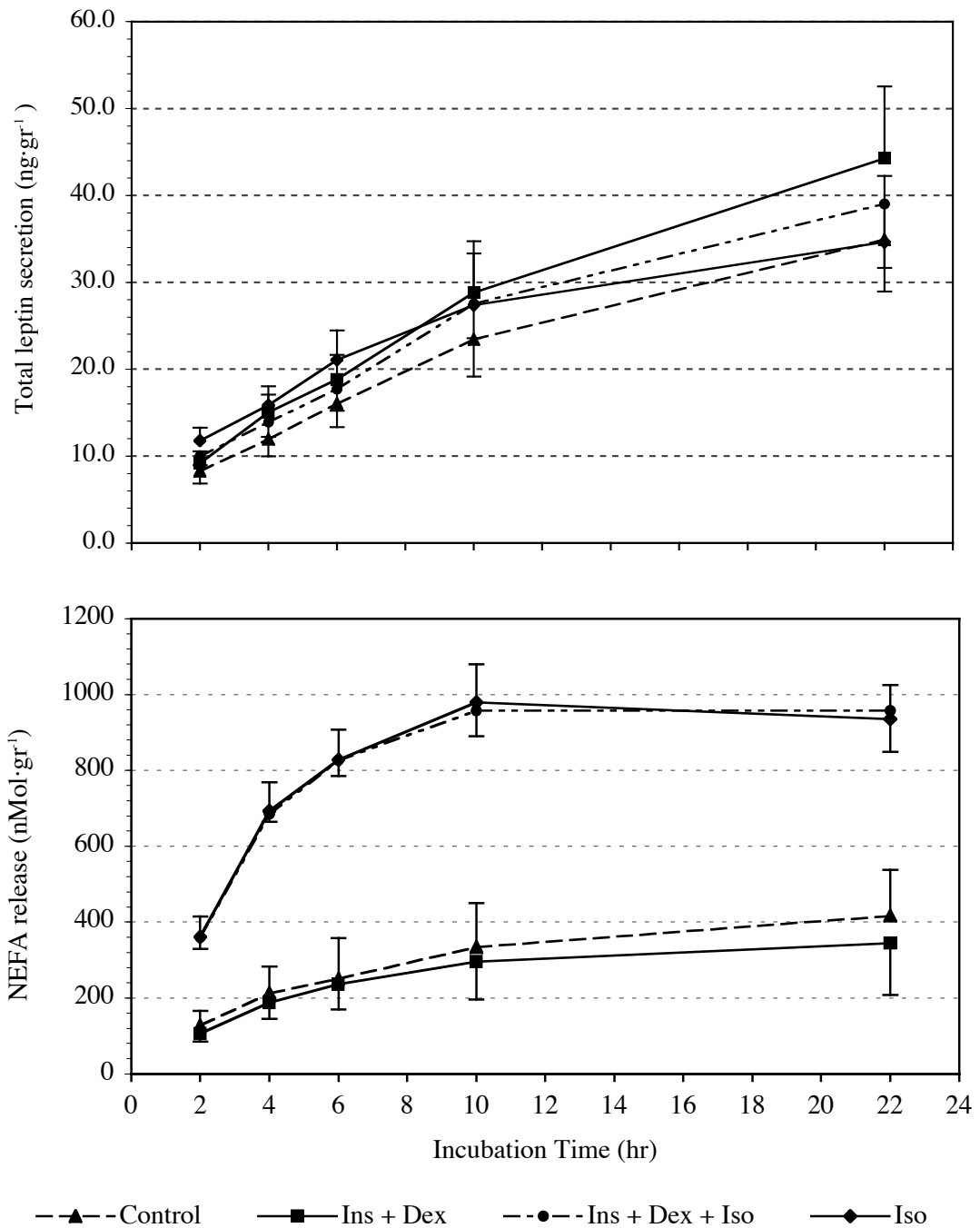


Figure 3.1 A and B: Time course of total amounts of leptin and NEFA released into the culture medium by human adipose tissue fragments. Fragments were incubated for 22 h under the following four conditions: control, insulin+dexamethasone, insulin+dexamethasone+isoprenaline and isoprenaline. Aliquots of culture medium were collected at five points in time during the incubation period and analysed for leptin and NEFA concentration. Leptin secretion was not affected by the culture medium condition ($P>0.05$). Isoprenaline showed to be a very potent stimulus of NEFA release ($P<0.05$). Data are means and standard errors of the mean.

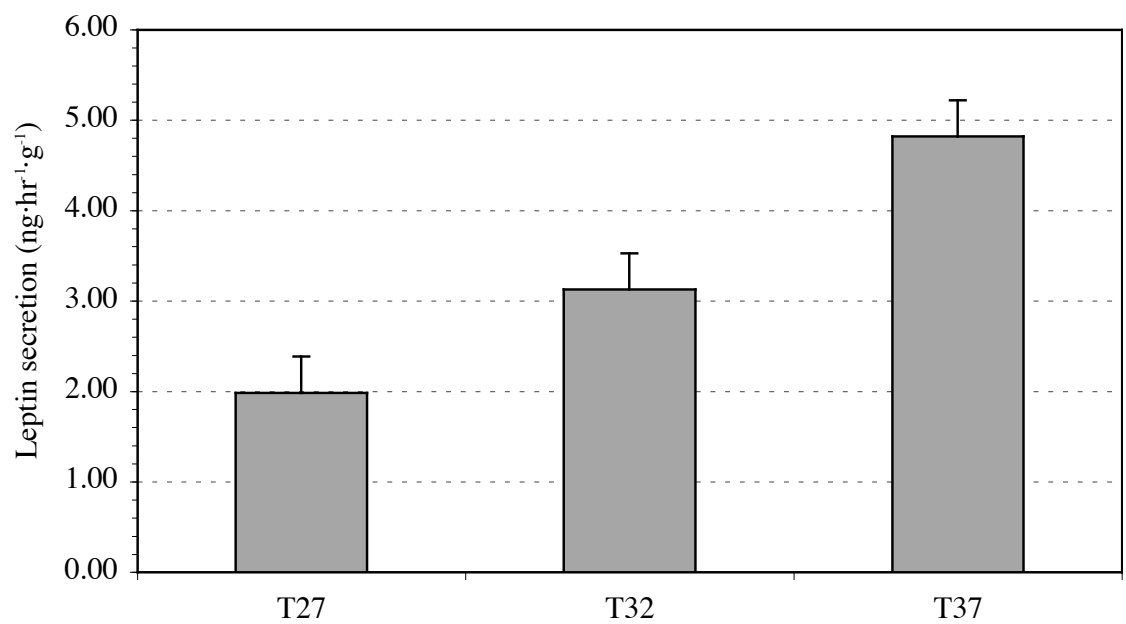


Figure 3.2: Direct effects of incubation temperature on leptin secretion from human adipose tissue fragments into the culture medium. Fragments were incubated in water baths set at three different temperatures (27, 32 and 37°C). Data are means and standard errors of the means ($P<0.05$; $n=8$).

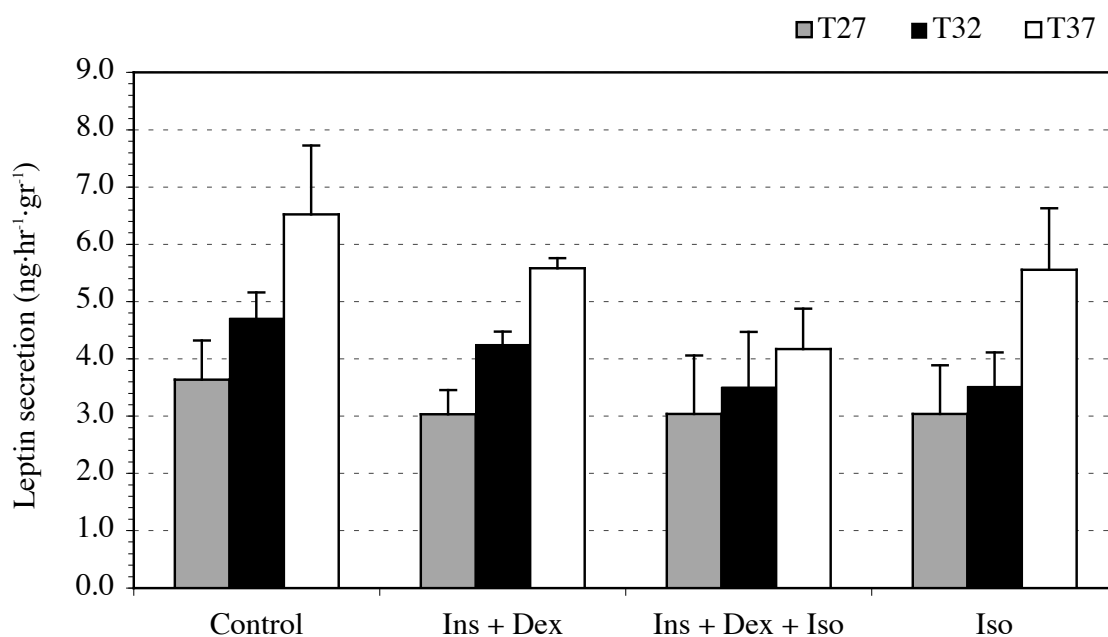


Figure 3.3: Hourly rate of leptin release into the culture medium by human adipose tissue fragments during the first six hours of incubation (data are means and standard error of the means). Human adipose tissue fragments were incubated at three different temperatures (27, 32 and 37°C) under four different conditions (control, insulin+dexamethasone, insulin+dexamethasone+isoprenaline and isoprenaline, n=3). Supplementation of the culture medium did not effect leptin secretion ($P>0.05$), however the direct temperature effects were apparent in all condition ($P<0.05$).

3.3.3 Temperature and Hormonal Effects on Glucose Uptake

Glucose concentration of the culture medium decreased over time ($P < 0.05$), indicative of glucose uptake by the adipose tissue fragments. Average glucose uptake by subcutaneous adipose tissue fragment after 22 h of incubation was $28.7 (\pm 1.1) \mu\text{mol} \cdot \text{gr}^{-1}$ of tissue. Figure 3.4 shows the hourly rate glucose uptake by adipose tissue fragments during the first six hours of incubation for each condition and incubation temperature. The amount of glucose uptake was significantly affected by the incubation temperature and by the supplementations in the medium (P 's < 0.05). Glucose uptake by the fragments was greater at the higher incubation temperatures. The supplementation of the culture medium with either insulin/dexamethasone, isoprenaline or the combination of all three increased glucose uptake human adipose tissue fragments, particularly when fragments were incubated 37°C .

3.3.4 Temperature and Hormonal Effects on Non-Esterified Fatty Acids Release

Figure 3.5 shows the average hourly rate of NEFA release during the first six hours of incubation for the four conditions. NEFA release by human adipose tissue fragments was not affected by the incubation temperature ($P > 0.05$). As expected, stimulation of the beta-adrenergic receptors by isoprenaline (alone and in combination with insulin and dexamethasone) showed to be a very potent stimulant of the release of NEFA ($P < 0.05$). This resulted in approximately four times higher NEFA concentrations in the culture medium when isoprenaline was present.

3.4 DISCUSSION

The presented *in vitro* studies were designed to quantify local and direct effects of temperature on leptin secretion from human subcutaneous adipose tissue. The principle finding is that leptin secretion is reduced at lower temperature. In the previous chapter it has been described how these *in vitro* results can be incorporated in a compartmental model of the interaction between environmental temperature and leptin. The presented model evaluated the local impact of reduced subcutaneous adipose tissue temperature on decreases in circulating leptin during cold exposure in humans. The results indicated that besides sympathetic regulation of leptin secretion, locally-

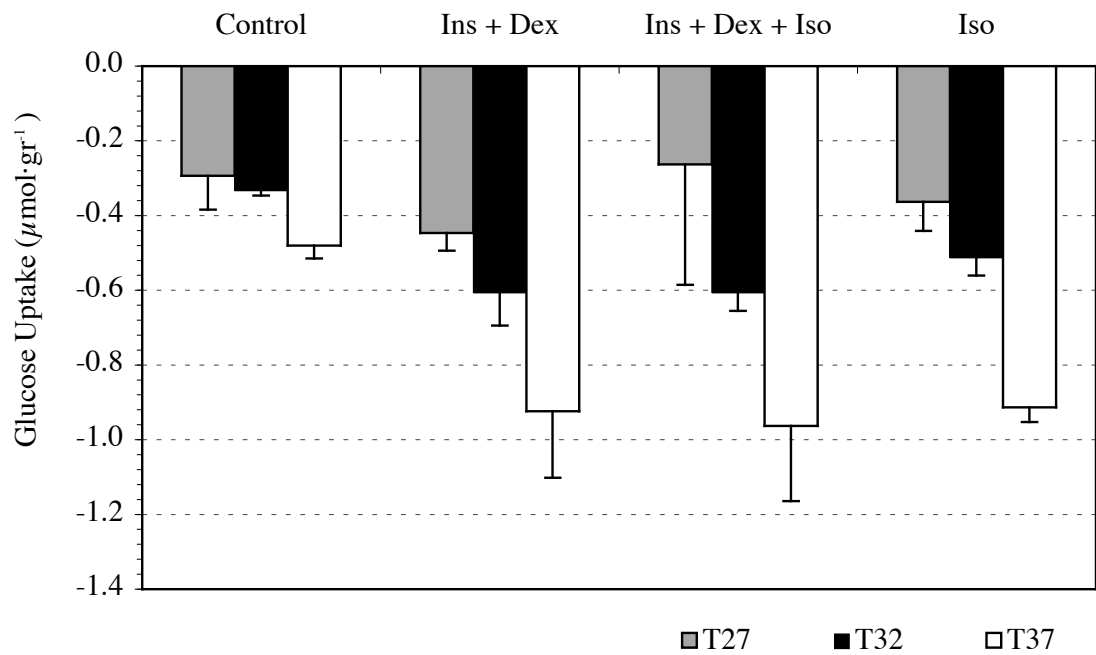


Figure 3.4: Hourly rate of glucose uptake from the culture medium by human adipose tissue fragments during the first six hours of incubation (data are means and standard error of the means). Human adipose tissue fragments were incubated at three different temperatures (27, 32 and 37°C) under four different conditions (control, insulin+dexamethasone, insulin+dexamethasone+isoprenaline and isoprenaline, n=3). Supplementation of the culture medium did not effect leptin secretion ($P>0.05$), however direct temperature effects on glucose uptake were apparent in all conditions ($P<0.05$).

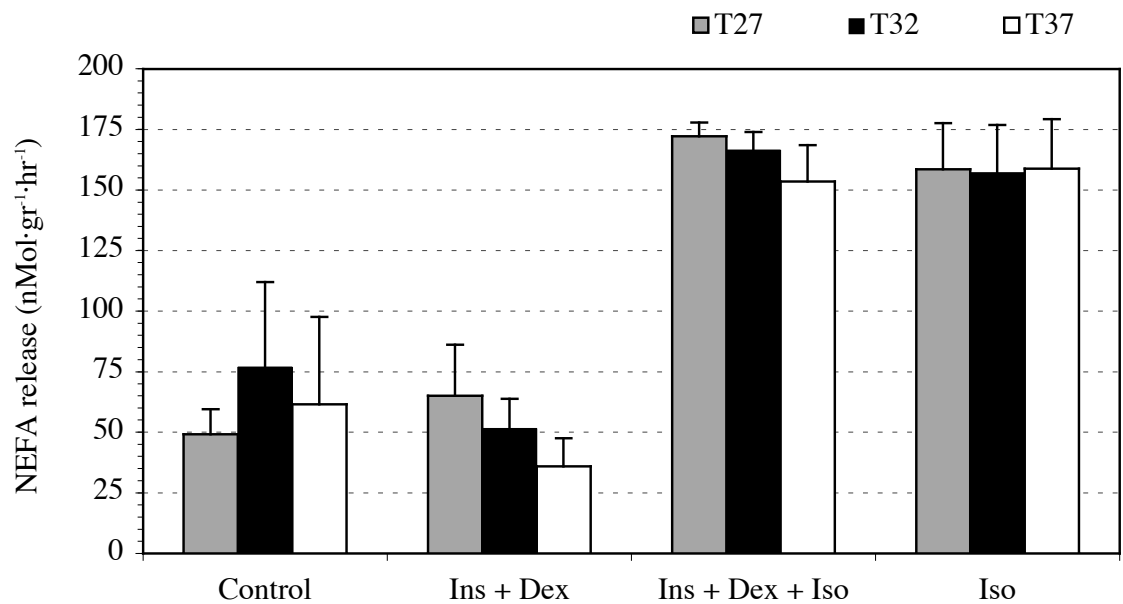


Figure 3.5: Hourly rate of NEFA release into the culture medium by human adipose tissue fragments during the first six hours of incubation (data are means and standard error of the means). Human adipose tissue fragments were incubated at three different temperatures (27, 32 and 37°C) under four different conditions (control, insulin+dexamethasone, insulin+dexamethasone+isoprenaline and isoprenaline. n=3). Incubation temperature did not affect NEFA release ($P>0.05$). Isoprenaline increased NEFA release from human adipose tissue fragments ($P<0.05$).

mediated effects of temperature can fully account for cold-induced decrease found in *in vivo* studies.

3.4.1 Leptin Secretion from Human Subcutaneous Adipose Tissue Fragments

Under the control condition (without hormones supplemented) total leptin secretion after 22 h was $49.5 (\pm 12.5) \text{ ng}\cdot\text{gr}^{-1}$ of tissue. This level of leptin secretion is comparable to the total amount of leptin secretion after 24 h by human subcutaneous adipose tissue reported by Ricci and Fried, which was approximately $50 \text{ ng}\cdot\text{gr}^{-1}$ (Ricci and Fried, 1999). Leptin secretion rate decreased over the incubation period from $4.3 (\pm 0.5) \text{ ng}\cdot\text{hr}^{-1}\cdot\text{gr}^{-1}$ of tissue in the first 2 h to $1.7 (\pm 0.5) \text{ ng}\cdot\text{hr}^{-1}\cdot\text{gr}^{-1}$ in the last 12 h of the 22 h incubation period. To our knowledge, no other study determined leptin secretion at five different time points within a period of 24 h of incubation. The presented results show that leptin secretion is linear in the first 10 h of incubation, while decreasing in the second half of the incubation (Figure 3.2 and 3.3).

3.4.2 Temperature Effects on Leptin Secretion

Direct and local effects of temperature on leptin physiology have also been investigated (Peino *et al.*, 2000). The authors demonstrated significant effects of incubation temperature on leptin secretion using human omental adipose tissue. The presented results are in agreement with their results, which show also a substantial reduction in leptin secretion at 32°C compared to 37°C (-68% , $P < 0.05$). Besides leptin secretion in the culture medium, Peino *et al.* also investigated the temperature effect on *ob* gene expression. Unfortunately, the authors do not provide any numeric data (in arbitrary units) regarding the densitometric analysis of the RT-PCR products of their 38 samples. Instead, only one picture of the ethidium bromide stained PCR products of one single sample is provided. However, based on their results the authors conclude that the decrease in leptin secretion rate was not accompanied by parallel changes in *ob* mRNA expression. This would indicate that a post-transcriptional mechanism underlies the temperature effects on leptin physiology. The concept of a post-transcriptional mechanism is supported by findings of Bradley *et al.* (2001) who demonstrated that while leptin secretion is basically reduced to zero at 20°C , intracellular leptin

concentration was significantly increased at this temperature compared to an incubation temperature of 37°C.

3.4.3 Hormonal Regulation of Leptin Secretion

Leptin secretion was not significantly affected by supplementation of the culture medium with insulin and dexamethasone and/or isoprenaline. While stimulatory effects of insulin and dexamethasone and inhibitory effects of isoprenaline on leptin secretion have previously been suggested, not all other *in vitro* studies have succeeded to demonstrate these effects. The results seem to depend on species, incubation period and culture conditions used as summarised in Table 3.2.

The length of the incubation period seems to be an important determinant for the effect of the supplementation. Kolaczynski *et al.* (1996) showed that stimulatory effects of insulin on *ob* mRNA expression could only be detected after 72 h of incubation. This increase in *ob* gene expression subsequently resulted in a significant increase in leptin secretion after 96 h. The results of Casabiell (2000) and others show even an inhibitory effect of insulin on leptin secretion during the first 48 h of incubation, while after 72 h of incubation leptin secretion was increased by insulin. These conflicting results of insulin on leptin may explain why the presented study did not show any significant effects of insulin and dexamethasone during the first 6 h of the 22 h incubation period.

The composition of the culture medium also may also influence leptin secretion. Most studies demonstrate dose-dependent effects of hormonal supplements in the culture medium (Considine *et al.*, 1997; Halleux *et al.*, 1998b). However, the results of Casabiell *et al.* clearly indicate that the effect of insulin on leptin secretion is affected by the glucose concentration of the culture medium self. At glucose concentrations of 6 mM and higher, there was a reduction of leptin secretion by insulin in the first 48 h, but after 72 h insulin stimulated leptin secretion. However, at 1 mM of glucose, leptin secretion continued to be reduced by insulin for the whole incubation period of 96 h (Casabiell *et al.*, 2000).

Finally, in the current study the stimulatory effect of supplemented insulin and dexamethasone may have been undetectable due to the stimulatory effect of the added

serum itself in the culture medium. It has been demonstrated that no leptin can be detected in 10% Foetal Bovine Serum (FBS) culture medium and this high concentration of FBS does not affect the detection of added standard leptin in the medium (Considine *et al.*, 1997). However, it has been suggested that other factors of the FBS may exert confounding effects on leptin secretion (Yoshida *et al.*, 1996).

3.4.4 Adipose Tissue Metabolism Responses to Temperature and Supplements

To quantify glucose uptake by adipose tissue fragments throughout the incubation period, glucose concentration of the culture medium was measured at six points in time. The glucose concentration significantly decreased over time and after 24 h of incubation the total glucose uptake amounted $27.7 (\pm 1.1) \mu\text{mol}\cdot\text{gr}^{-1}$. The amount of glucose uptake in this experiment is comparable to the level of glucose uptake of resting adipose tissue, measured using microdialysis ($24.2 \pm 6.5 \mu\text{mol}\cdot\text{gr}^{-1}\cdot 24\cdot\text{hr}^{-1}$) by Simonsen *et al.* (1994).

Glucose uptake by adipose tissue fragments was as expected significantly affected by the supplementations of insulin and dexamethasone and/or isoprenaline. These results are in agreement with Faulconnier *et al.* (2003), who also demonstrated that glucose uptake by ovine adipose tissue explants was higher when explants were incubated with insulin alone and together with dexamethasone compared to control. The concentrations of insulin ($2 \text{ mIU}\cdot\text{ml}^{-1}$) and dexamethasone (100 nM) and the incubation period (48 hrs) in the experiments of Faulconnier *et al.* were higher and longer than in the presented studies ($1.17 \text{ mIU}\cdot\text{ml}^{-1}$ and 25nM, 22 hrs, respectively).

The amount of NEFA's released into the culture medium increased over time incubation ($P<0.05$). However, after 10 h of incubation the rate of NEFA release had decreased almost zero. As expected isoprenaline demonstrated to be a potent stimulator of NEFA release. Incubation temperature did not have any effect on NEFA release. on lipolysis.

This investigation focussed on direct and local effects of temperature on leptin secretion and adipose tissue metabolism in human subcutaneous adipose tissue. The main observation was a significant reduction in leptin secretion at lower temperatures.

The previous chapter discussed how this observed local induced reduction on leptin secretion may account for the *in vivo* demonstrated decreases in circulating leptin during cold exposure. This study did not show any significant effects of insulin, dexamethasone and isoprenaline on leptin secretion from human adipose tissue fragments, or any significant interactions between temperature and the hormonal regulation of leptin secretion.

Leptin has demonstrated effects on satiety and energy expenditure, and is considered to play part in the feedback loop from the adipose tissue in the regulation of energy balance. Additionally, leptin receptors have been found in adipose tissue itself (Fruhbeck *et al.*, 1998) and *in vitro* studies suggest direct effects of leptin on adipocyte metabolism (Zhang *et al.*, 1999). It can therefore be concluded that cold-induced changes in leptin production and secretion may have significant implications on local adipose tissue metabolism and subsequently on body composition regulation.

CHAPTER 4: THE EFFECTS OF BETA-ADRENERGIC BLOCKADE ON PHYSIOLOGICAL RESPONSES TO COLD-WATER IMMERSION AND SUBSEQUENT REWARMING

4.1 INTRODUCTION

4.1.1 Sympathetic Activation During Cold Exposure

While most organs are simultaneously innervated by the post-ganglionic nerve endings of both the parasympathetic and sympathetic nervous system (SNS), white adipose tissue together (WAT) with blood vessels and sweat glands, are only under the control of the sympathetic nervous system (Snitker *et al.*, 2000). During cold-water immersion (10°C) direct sympathetic activation results in rapid increases in plasma norepinephrine concentration during the first 2 minutes and this continues to rise gradually till it reaches a maximum after approximately 45 min (Johnson *et al.*, 1977). This cold-induced increase in sympathetic nervous activity is thought to mediate several compensatory physiological responses to cold such as peripheral vasoconstriction and increased thermogenesis.

4.1.2 Cold-Induced Changes in Leptin Physiology

Cold-induced suppression of circulating leptin has been reported by various authors in rodent and ovine studies (Trayhurn *et al.*, 1995; Hardie *et al.*, 1996b; Asakuma *et al.*, 2003). Rodent studies also demonstrate reductions in relative *ob* mRNA abundance in both visceral and brown adipose tissue during exposure to cold air (4°C) for 2-24 h (Trayhurn *et al.*, 1995). Similarly human studies demonstrate reductions in circulation plasma leptin concentration in females during cold-air (6.3°C) exposure (Ricci *et al.*, 2000) and in cold-water immersed male subjects as described in Chapter 2.

Inhibitory effects of beta-adrenoreceptor agonists on both *ob* mRNA expression and circulating leptin concentration are reported in rodent and human studies (Trayhurn *et al.*, 1995; Giacobino, 1996; Deng *et al.*, 1997; Pinkney *et al.*, 1998; Ricci and Fried, 1999). These results led to the suggestion that the SNS is a major regulator of leptin

production (Giacobino, 1996; Trayhurn *et al.*, 1999; Rayner and Trayhurn, 2001). Subsequently, these results of sympathetic agonists together with the notion of increased sympathetic activation during cold led to the conclusion that cold-induced sympathetic activation plays a major role in the inhibitory effects of cold on leptin physiology (Deng *et al.*, 1997; Trayhurn *et al.*, 1999). However, these agonist studies demonstrate only the potential effects of sympathetic activation, rather than its specific involvement in cold-induced suppression of leptin physiology. To our knowledge there is only one study that used a beta-adrenergic antagonist during cold exposure. Evans *et al.* (1999) investigated the effects of beta₁, beta₂ and beta₃ receptor blockade on cold-induced modifications of serum leptin concentration and *ob* mRNA expression in WAT and brown adipose tissue (BAT) in mice. The interpretation of their results is open to opposition. In BAT, either propranolol (beta₁ and beta₂ antagonist) or SR 59230 (selective beta₃ antagonist) alone could not prevent cold-induced decreases in *ob* mRNA expression. The interaction of propranolol or SR 59230 on the temperature induced changes in *ob* mRNA expression in WAT and serum leptin seem to be opposite. In WAT *ob* mRNA expression was not significantly decreased after beta₃ receptor blockade by SR 59230, in contrast the decrease in serum leptin did not reach statistical significance after beta₁ and beta₂ receptor blockade by propranolol. When beta-adrenergic blockade was applied by both propranolol and SR 59230A, the applied t-tests did not demonstrate any statistical significant differences between 22°C and 4°C. The authors therefore concluded that the combination of beta₁, beta₂ and beta₃ receptor blockade prevented cold-induced decreases. This conclusion is based on the assumption that a failure to detect an effect is equivalent to demonstrating that no effect was present. It could also be argued that there was a tendency that after (selective) beta-adrenergic blockade, *ob* mRNA expression (WAT and BAT) and serum leptin concentration were still decreased after cold exposure. However, these decreases were only statistically significant in some but not all situations. It is likely that, more appropriate, factorial *ANOVA* analyses of the data would indicate that beta adrenergic blockade did not prevent cold-induced decreases in circulating leptin and *ob* mRNA expression in either BAT and WAT.

To our best knowledge, in humans the involvement of beta-adrenergic receptors in reductions in plasma leptin during cold-exposure has not previously been

investigated. The aim of the current investigation is therefore to determine whether beta-adrenergic blockade in humans prevents cold-induced changes in circulation leptin.

4.1.3 Beta-adrenergic Regulation of Leptin

In rodents the beta₃-adrenergic receptor seems to be the predominant beta-receptor involved in the regulation of leptin (Giacobino, 1996). In contrast, human studies seem to indicate that the beta-adrenergic regulation of leptin is mediated by other beta-receptors than beta₃-adrenergic receptor. First of all, several studies show no associations between polymorphism of the beta₃-adrenergic receptor Trp64Arg variant and circulating leptin concentration (Janssen *et al.*, 1998; Snitker *et al.*, 1998; Vendrell *et al.*, 1998). Secondly, Van Baak and colleagues investigated the acute effects of one single dose of the new beta₃ receptor agonist, L-796568, in obese men (2002). Administration of 1000mg of this beta₃ receptor agonist increased energy expenditure, plasma glycerol and FFA but did not affect plasma leptin concentration. And finally, results of longer-term administration of a beta₃-adrenoceptors agonist (CL 316243, 1,500 mg daily) in lean healthy young men also did not show any effect on circulating leptin concentration after 4 nor after 8 weeks (Weyer *et al.*, 1998). Therefore, it seems that beta₃-adrenergic receptors do not play a significant role in the human regulation of leptin.

This is also confirmed by *in vitro* studies on beta-adrenergic regulation of leptin secretion. Scriba *et al.* (2000) investigated the relative effects of stimulation of the three beta receptors on leptin secretion from cultured human subcutaneous adipocytes. As expected stimulation of all three beta-receptor increased glycerol release into the culture media in a similar manner. Leptin secretion was markedly reduced by isoproterenol (non-selective beta-adrenergic agonist) and to a lesser extent by dobutamine (beta₁-selective) and fenoterol (beta₂ selective). However, none of the beta₃ selective agonists (BRL 37344 and CGP 12177) affected leptin secretion. Therefore, on the basis of both the *in vivo* and *in vitro* studies, it can be concluded that in humans the sympathetic regulation of leptin is predominantly through the beta₁ and beta₂ adrenergic receptors, rather than by the beta₃-adrenergic receptor.

4.1.4 Cardiovascular Responses to Cold-Exposure and Rewarming

When people are exposed to cold, blood flow to the periphery is reduced to limit heat loss from the body to the environment. These changes in vasomotor tone depend primarily on norepinephrine release and alpha-adrenoreceptors induced vasoconstriction in the cutaneous vasculature (Frank *et al.*, 2000). The combined result of cold-induced and hydrostatically induced redistribution of peripheral blood during cold-water immersion leads to an increased central blood volume, which subsequently increases stroke volume. Cardio-respiratory baroreceptor stimulation results from the increased stroke volume, which results in reduced cardiac frequency (f_c), *i.e.* bradycardia as typically observed during cold-water immersion. However, if the extent of the cold stress is such that it induces significant shivering, f_c will increase as a result of the physical activity involved with shivering (Simeckova *et al.*, 2000). The f_c response to cold-water immersion is therefore mainly determined by the degree of cold stress that is applied (Sramek *et al.*, 2000). When cold-exposure is followed by rewarming, cutaneous blood flow will initially increase as a result of local effects of temperatures on vascular smooth muscle followed by sympathetically driven vasodilatation mediated by beta adrenergic receptors (Savard *et al.*, 1985).

4.1.5 Thermoregulatory Responses to Cold-Exposure and Rewarming

Body temperature reflects the total balance between heat accumulation (metabolic heat production and environmental heat exchange) and heat dissipation. During cold exposure heat loss by the body is reduced by cold-induced vasoconstriction. However, due to the large thermal gradient between the body and the surrounding, body heat is lost by thermal conduction and body temperatures decrease as a result. Since water has a great thermal conductivity skin temperatures (T_{sk}) rapidly decrease during cold-water immersion. In some cases, a paradoxical increase in core temperature is seen during the early phase of immersion (Tikuisis *et al.*, 1988). This represents the transient decrease in body heat loss due to cold-induced cutaneous vasoconstriction. Various studies have shown that body fat and in particular subcutaneous adipose tissue provides significant protection during cold exposure. Significant negative correlation has been demonstrated between estimates of body fat

percentage (sum of skinfolds) and decrease in core temperature during cold exposure (Prisby *et al.*, 1999).

When warm air is used to rewarm a person from mild hypothermia, the thermal gradient between the skin and surrounding is reversed and heat is gained by conduction, convection and radiation, resulting in rapid increases in T_{sk} . As a result of the circulatory changes at the periphery (i.e. vasodilatation), as well as continuation of core to skin thermal conduction, core temperature may continue to decrease until the core-to-skin thermal gradient is reversed, a phenomenon referred to as the afterdrop (Webb, 1986).

4.1.6 Thermogenesis

During cold-exposure heat production (thermogenesis) increases initially by non-shivering pathways and later, when body temperature decreases further, by shivering. The first, non-shivering thermogenesis, also known as cold-induced thermogenesis or chemical thermoregulation, is thought to be under beta-adrenergic control (Jansky *et al.*, 1997; Simeckova *et al.*, 2000, Jansky 2002). This is supported by the observation that the beta-adrenergic antagonists, propranolol, decreased cold-induced increases in oxygen uptake in pigs exposed to 15°C air (Dauncey and Ingram, 1979).

Studies of the relative contributions of carbohydrates and lipids in cold-induced thermogenesis (non-shivering and shivering) show conflicting results (Jessen, 1980; Tikuisis *et al.*, 1988; Martineau and Jacobs, 1989; Vallerand and Jacobs, 1989; Jacobs *et al.*, 1994). Vallerand and Jacobs (1989) determined substrate utilization by indirect calorimetry and concluded that carbohydrates was the major substrate source during cold exposure. In contrast, Jessen *et al.* (1980) suggested based on significant increases in plasma non-esterified fatty acids (NEFA) concentration (96%) and relatively small reduction of plasma concentration of glucose (-6%), that lipid stores in adipose tissue are the major substrate for the increased energy demand during cold exposure. These discrepancies may be explained by differences in methods used to determine substrate utilisation, the magnitude of cold stimulus applied and thus the shivering intensity (level of physical activity), cooling protocol and or nutritional state of the subjects.

Haman *et al.* (2002) performed a thorough study using a combination of stable isotope and indirect calorimetry methods to determine the relative contribution of plasma glucose, muscle glycogen, and lipid oxidation during 2 h of cold exposure with low intensity shivering. The results indicate that lipids are the major fuel source during cold-exposure. According to their results lipids contributed 50%, muscle glycogen 30%, plasma glucose 10% and protein also 10% to total heat production.

Subcutaneous adipose tissue is, besides being a thermal insulator, the largest storage area for triglycerides. The role of subcutaneous adipose tissue in cold-induced lipolysis has been investigated (Koska *et al.*, 2002). Local lipolysis was evaluated during cold air exposure (4°C for 30 min) by measuring interstitial glucose and glycerol concentrations using a microdialysis probe in a subcutaneous adipose tissue depot and circulating concentrations of the same substances. Cold exposure significantly increase glycerol concentrations in both plasma and dialysate while there where no difference in either glucose concentrations. These studies support a significant role of subcutaneous adipose tissue in cold-induced thermogenesis.

4.1.7 Summary

Cold exposure increases sympathetic nervous activity, which is thought to mediate several compensatory physiological responses to cold such as peripheral vasoconstriction and increased thermogenesis. Moreover, cold-induced decreases in circulating leptin and *ob* gene expression have been reported in various studies and are often ascribed to increased sympathetic outflow or more specifically to the subsequent stimulation of the beta-adrenergic receptors (Rayner and Trayhurn, 2001). However, the specific involvement of the beta-adrenergic pathway in cold-induced decreased in circulating leptin has not previously been investigated in humans.

During cold exposure subcutaneous adipose tissue fulfils the dual function of thermal insulator and major fuel source for cold-induced thermogenesis. Stimulation of beta-receptors during cold exposure results in increased lipolysis and circulating NEFA concentration. While the thermoregulatory and cardiovascular responses during cold-water immersion are well documented, there is limited data on how these variables

respond to subsequent rewarming. Additionally, data on the role of beta-receptors in vasodilatation during rewarming is limited.

Propranolol is a non-selective beta-adrenergic receptor blocking agent and has no other autonomic nervous system activity. Peak plasma concentrations of propranolol are attained 60 to 90 minutes after administration of Inderal tablets and the plasma half-life is 2 to 3 hours (MIMS, 2004). Therefore, administration of propranolol one hour before immersion results is appropriate to investigate the effects of beta-adrenergic blockade on physiological responses during 60 min of cold-water immersion and subsequent 60 min of rewarming.

The present study was designed to investigate beta-adrenergic involvement in changes in circulating leptin and thermal and cardiovascular responses during cold-water immersion and subsequent rewarming. In this study, thermoregulatory and cardiovascular responses and circulating levels of leptin, glucose and NEFA were measured during cold-water immersion and subsequent rewarming. Each subject underwent two trials, one with and one without the beta-adrenergic antagonist propranolol.

4.2 METHODS

4.2.1 Subjects

Fourteen healthy subjects (seven females, seven males) participated in this study. The Human Research Ethics Committee of the University of Wollongong approved the study. Each subject received a Subject Information Package, provided consent and satisfactorily completed a medical screening questionnaire. Each subject underwent two separate trials, one with and one without, the beta-adrenergic antagonist, propranolol. In each trial, subjects firstly underwent 60 min of cold exposure in an immersion tank [Control: $18.35 (\pm 0.12)^{\circ}\text{C}$; Propranolol: $18.28 (\pm 0.13)^{\circ}\text{C}$, $P > 0.05$], followed by 60 min of rewarming in a heated climate chamber [Control: $38.35^{\circ}\text{C} (\pm 0.21)^{\circ}\text{C}$; Propranolol: $38.04^{\circ}\text{C} (\pm 0.21)^{\circ}\text{C}$, $P > 0.05$].

4.2.2 Procedures

Subjects presented at 0800 h, after an overnight fast, for blood sampling and anthropometric measurements and consumed a standard breakfast (38 kJ·kg⁻¹ plus 5 ml·kg⁻¹ fluid). Instrumentation of the subjects started at 1100 h and was followed by a rest period of at least 30 min until the start of immersion. Propranolol (80 mg Deralin: Alphapharm Pty. Ltd, Australia) was administered orally 60 min prior to the start of immersion. The study procedures are summarized in Figure 4.1.

4.2.2.1 Experimental Standardisation

Subjects were asked to refrain from strenuous exercise and to abstain from the consumption of food, alcohol and caffeine from 12 h before each experiment to ensure that testing was performed with minimal influence from external factors. This information was documented in the subject information package. Furthermore, trials commenced at the same time (between 1200 and 1230 h), in order to control for circadian shifts in measured variables such as core temperature (T_c). This time period also coincided with the period of minimal influence of diurnal rhythm on plasma leptin concentration (Sinha *et al.*, 1996a).

4.2.3 Cold-Water Immersions.

The immersion tank was the same as described in Chapter 2. Subjects were positioned in the stainless steel water tank by an electrical hoist and immersed until the water level reaching their fourth intercostal space. The depth of immersion was dictated by the requirement to have subject arms out of the water to allow for blood sampling. Both the arms of the subject were supported by a wooded tray across the tank.

4.2.3.1 Subject Preparation

Subjects presented at 0800 h after an overnight fast. Following a 10 min seated rest, a butterfly needle was inserted into the antecubital vein of the left arm and 10 ml of blood was collected. Subsequently anthropometric measurements, as mass, height and skin fold thicknesses were obtained. The standard breakfast was then provided.

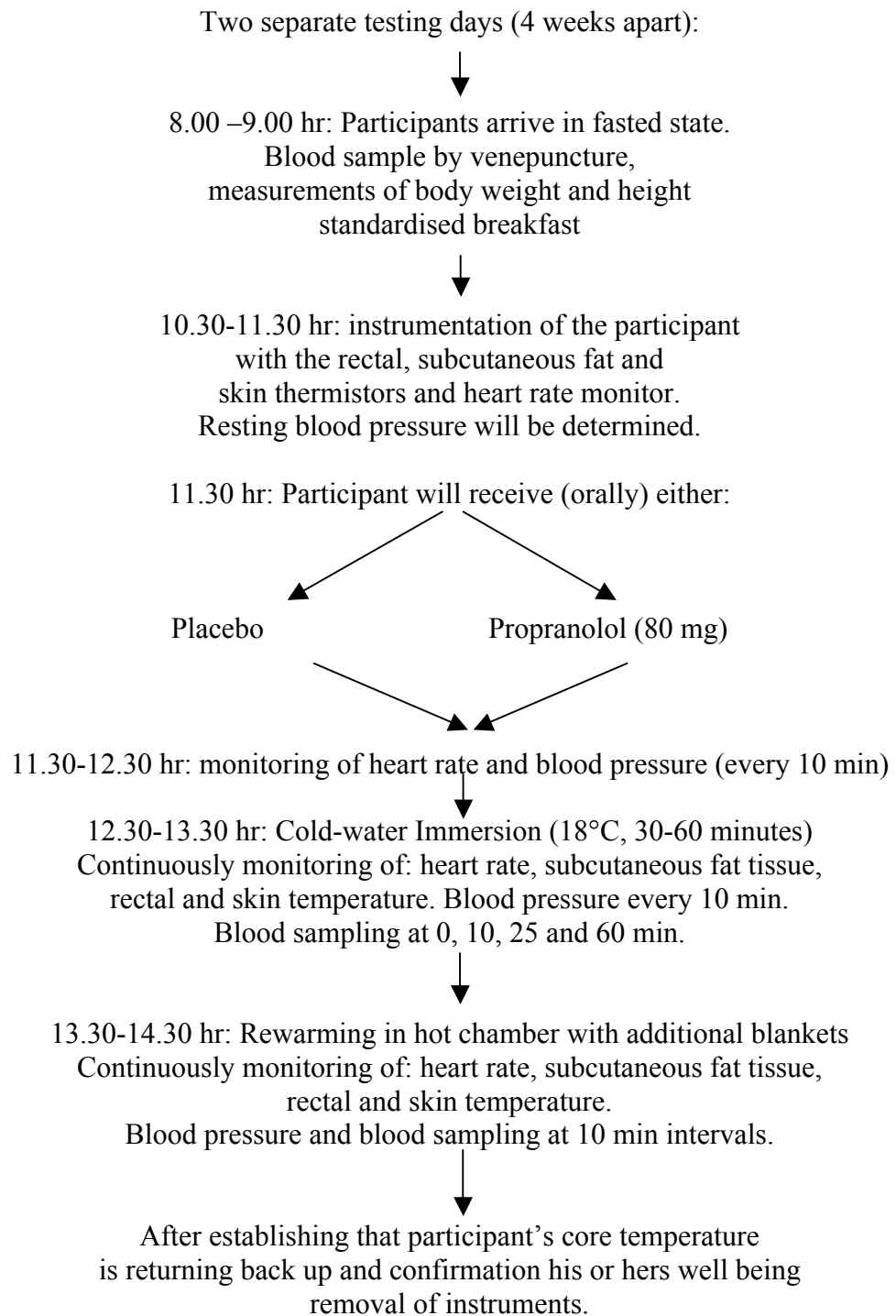


Figure 4.1: Study procedures of cold-water immersion and subsequent rewarming experiments with and without beta-adrenergic receptor blockade by 80 mg of propranolol.

The subject consumed the provided food and drink items and rested (usually reading) until approximately 1100 h. One hour prior to immersion, subjects were instrumented with the equipment for temperature and heart rate measurement. This equipment included; rectal probe, eight skin thermistors, four subcutaneous thermocouples and a cardiac frequency monitor. Subjects inserted their own rectal probe, for the measurement of rectal temperature (T_{re}) to a predetermined depth of 12 cm. A cardiac frequency monitor was fitted around the chest and skin thermistors were attached at eight sites with a single layer of waterproof tape, with the dimension of approximately 5 by 5 cm. A catheter was inserted by a registered nurse in the antecubital vein of the right arm. Pre-immersion data was collected with the subjects seated at rest beside the immersion tank. Just prior to immersion, blood was collected for pre-immersion determinations of leptin, glucose and NEFA concentrations. Following pre-immersion data collection, subjects were transferred quickly to the immersion tank for commencement of the cold exposure period. After 60 min in the immersion tank, subjects were quickly transferred in a wheelchair into a heated climate chamber for a 60 min rewarming period. Thermal and cardiac data were collected continuously during the immersion and subsequent rewarming period. Blood samples were collected after 25 and 60 minutes of immersion and every ten minutes during rewarming.

4.2.4 Apparatus

4.2.4.1 Body Temperature

Rectal temperature was recorded using a thermistor, (YSI type-401, Yellow Springs Instrument Co. Inc., Yellow Springs, OH, U.S.A.) inserted 12 cm beyond the anal sphincter. Measurements were recorded at 0.067 Hz using a data logger (1206 Series Squirrel, Grant Instruments, Ltd., Cambridge U.K.). After completion of the experiments, data was down loaded onto a computer for storage and subsequent analysis.

4.2.4.2 Skin Temperature

Skin surface temperatures were collected at eight sites (EU type, Yellow Springs Instruments Co. Inc., Yellow Springs, OH, U.S.A.). These sites included: forehead,

right scapula, left upper chest, right upper arm, left forearm, left hand, right anterior thigh and left calf. Mean skin temperatures (T_{sk}) was derived using an area-weighted summation (ISO, 1992). Temperatures were also sampled at 15-s intervals (1206 Series Squirrel, Grant Instruments Ltd., Cambridge, U.K.).

4.2.4.3 Subcutaneous Temperature

Temperature probes (Physitemp Instruments Inc. Clifton, NJ, U.S.A.) consisting of a thin and flexible Teflon catheter, containing one thermocouple, were positioned into the subcutaneous adipose tissue layer of the abdomen, thigh, upper arm and back. The sterilised thermocouples were inserted, through a skinfold pulled away from the underlying muscle layer using a sterile 18-G hypodermic needle as a guide. After insertion, the depth of the guide needle was determined using a micrometer to measure the layer of skin and adipose tissue above the needle. Subsequently, the needle was withdrawn, leaving the thermocouple at this depth until post-experimental removal. Skinfold thickness at the four sites was determined (Harpenden Eiyoken-type, Maikosha Co Ltd., Tokyo, Japan) to provide an estimation of the subcutaneous adipose tissue layer of each site.

4.2.4.4 Cardiac Frequency

Cardiac frequency (f_c) was recorded from ventricular depolarisation (Model PE3000, Polar Electro Sport Tester, Kempele, Finland) and also sampled at 15-s intervals. Cardiac frequency, determined using this system, has been validated in our laboratory against a five lead electrocardiogram (Quinton, Q5000).

4.2.4.5 Forearm Blood Flow

Forearm blood flow (Q_{sk}) data was collected using venous-occlusion plethysmography (EC4 Plethysmograph, D.E. Hokanson Inc., U.S.A.). The measurement relies on the occlusion of forearm venous return, without changing arterial inflow. This is achieved by inflating a venous-occlusion cuff, placed proximal to the left elbow, to a pressure of 50 mmHg. Due to the variability of hand blood flow a second cuff was inflated at the left wrist to 160 mmHg to prevent flow and to remove any

artefact. The cuff was automatically inflated for 8 s and deflated for 12 s. Analog output from the plethysmography system, sampled at 20 Hz, was passed, via an eight channel, 12-bit analog-to-digital converter (Computer Boards inc. PPIO-A18, Mansfield, U.S.A.), to an IBM compatible laptop computer (Total Peripherals, Notebook 386SX, Sydney, Australia). Forearm blood flow data was collected once prior to immersion and every 15 min during rewarming.

4.2.4.6 Skin Blood Flow

Skin blood flow was determined using Laser-Doppler velocimetry (Vasamedics Inc., TSI Laserflo BPM², U.S.A). This method depends on the Doppler shift of laser light reflected by moving particles. The laserflo BPM² incorporates a gallium aluminium arsenide semiconductor laser, operating in a single longitudinal mode, with a wavelength of 780 nanometers. The fibre optic probe has two receiving fibres with a core diameter of 100 microns, a numerical aperture of 0.28, and a spacing of 0.5 mm from the transmitting fibre, which has a core diameter of 50 microns and a numerical aperture of 0.20. Prior to each test the integrity of the fibre optic probe and the electrical zero of the system were checked. Analog output was passed, via an eight channel, 12-bit analog-to-digital converter (Computer Boards Inc., PPIO-A18, Mansfield, U.S.A.), to an IBM compatible laptop computer (Total Peripherals, Notebook 386SX, Sydney, Australia). Skin blood flow was simultaneously collected with forearm blood flow prior to immersion, under neutral-warm conditions, and during the rewarming period every 15 minutes.

4.2.4.7 Mass, Height and Skinfolds

Mass was determined on each test day (Model No fw-150k, AND, Milpitas, CA, U.S.A.) with subjects wearing only a swimming costume. Height was determined using a stadiometer (Holtain Ltd., Britain). Skinfold thicknesses were measured at seven sites (triceps, biceps, subscapular, supraspinale, mid-abdominal, medial calf and mid-thigh: Harpenden, Eiyoken-type, Maikosha Co Ltd., Tokyo, Japan), with adiposity represented as the sum of seven skinfolds on the first test day.

4.2.5 Blood Analyses

Blood samples were taken in fasted subjects upon arrival and prior to immersion, after 25 and 60 min of immersion, and every 10 min during rewarming. Samples were kept on ice and centrifuged within 20 min (2000 g), with aliquots of separated plasma and serum stored at -80°C for subsequent analysis.

4.2.5.1 Leptin

Plasma leptin concentration analyses were performed, in duplicate, by radioimmunoassay technique. Circulating leptin concentration of lean males as those who participated in the study have been reported to be $<5\text{ng}\cdot\text{ml}$, therefore a sensitive human leptin radio immuno assay kit (SHL-81K, Linco Research, St. Charles, MO, U.S.A.) was used. The sensitivity of the kit was $0.05\text{ ng}\cdot\text{ml}^{-1}$.

4.2.5.2 Non-Esterified Fatty Acids and Glucose

Circulating glucose and non-esterified free fatty acid concentrations were determined using enzymatic, colorimetric methods (NEFA C, Wako Pure Chemical Industries Ltd., Osaka, Japan; Glucose HK, Roche Diagnostics Pty. Ltd., Castle Hill, Australia). Samples were processed on a COBAS Mira Plus system (Roche Diagnostics Pty. Ltd., Castle Hill, Australia).

4.2.6 Design and Analyses

The study was based on a repeated-measures design (2 within factors). Cold-water immersion and rewarming data were analysed using similar but separate statistical tests. A problem that may arise when using repeated *ANOVA* design is that the repeated measures effects are often not independent of each other, such that changes over time are correlated across subjects. When this occurs, the compound symmetry and sphericity assumptions of the repeated measures *ANOVA* are violated and analysis may not be valid. Data was therefore analysed using a multivariate approach to repeated measures Analysis of Variance (*MANOVA*) as this analysis bypasses the assumption of compound symmetry and sphericity altogether.

A significant interaction between time and treatment effect (time*treatment) indicates that the response dynamics of the particular variable to the thermal stress were not parallel in control and propranolol condition. For each variable for which a significant interaction was found, data of the two treatment conditions were analysed separately.

Post-hoc tests by means of paired t-test were performed to determine differences in the magnitude of the response between the control and propranolol group.

Thermal and cardiac frequency data were averaged for the 2 min prior to each 10 min interval, such that values were derived for each 10-min period from 0 to 60 min of exposure. The chosen statistical analysis, however, allowed incorporation of only four time points per trial test due to the degrees of freedom 'consumed' by the multivariate approach.

Many studies have shown that plasma leptin concentrations, especially in a sample population including both genders, are not normally distributed, and are therefore log-transformed. The current sample size of 14 participants (7 males, 7 females) is too small to test the distribution of plasma leptin concentration. Therefore, statistical analyses were performed on both raw and log-transformed data.

Alpha was set at the 0.05 level for all analyses. Data for all dependent variables are presented as means with standard errors of the means unless otherwise indicated.

4.3 RESULTS

All subjects completed the cold-water immersions, without any signs of distress or feeling unwell. During the rewarming period of the blockade trials however, two female subjects reported to feel faint and showed mild hypotension. In both instances, the subjects were immediately placed in a supine position and continuously monitored by the medical staff. The data collected during the rewarming period for these subjects were excluded from the statistical analysis.

4.3.1 Baseline Comparisons between Control and Partial Beta-adrenergic Blockade Trials.

No significant differences between the control and blockade trial were observed in body weight [74.0 (\pm 3.4) kg versus 74.1 (\pm 3.4) kg; $P>0.05$] and in the sum of skinfolds [154 ± 16 mm compared to 150 ± 14 mm; $P>0.05$].

Body temperatures at baseline, also shown in Table 4.1 were also not different between control and blockade trials. Baseline temperature values match those expected in the resting state of normal healthy subjects. Mean baseline cardiac frequency was 75.1 (\pm 1.9) in the control trials $\text{b}\cdot\text{min}^{-1}$ and 74.9 (\pm 1.7) $\text{b}\cdot\text{min}^{-1}$ in the blockade trials ($P>0.05$). These resting values are slightly elevated from those expected in a healthy and predominantly trained population. This is probably due to fact that the subjects were not completely rested and slightly anticipated the cold-water immersion.

There were no significant differences between the control and propranolol trials in fasting NEFA, glucose and leptin concentrations (see Table 4.1). The fasting values of glucose fell all within the “normal” fasting ranges of 4.0-6.0 $\text{mMol}\cdot\text{L}^{-1}$. Approximately 3 h after subjects had consumed their breakfast, glucose concentration had returned to fasted baseline value in both conditions [Control: 4.40 (\pm 0.14) versus 4.60 (\pm 0.22) $\text{mMol}\cdot\text{L}^{-1}$; $P>0.05$, Propranolol: 4.47 (\pm 0.09) versus 4.68 (\pm 0.34) $\text{mMol}\cdot\text{L}^{-1}$; $P>0.05$]. Circulating NEFA concentration after breakfast and prior to immersion was significantly lower than the fasted level early in the morning [Control: 349 (\pm 48) versus 87 (\pm 32) $\text{nMol}\cdot\text{L}^{-1}$, $P<0.05$, Propranolol: 374 (\pm 50) versus 37 (\pm 6) $\text{nMol}\cdot\text{L}^{-1}$; $P<0.05$]. In both conditions, fasting plasma leptin concentration at baseline was significantly higher than prior to immersion [Control: 8.30 (\pm 2.06) versus 6.74 (\pm 1.54) $\text{ng}\cdot\text{ml}^{-1}$; $P<0.05$, Propranolol: 7.77 (\pm 1.76) versus 6.47 (\pm 1.28) $\text{ng}\cdot\text{ml}^{-1}$; $P<0.05$]. This decrease in plasma leptin in the morning is consistent with the reported diurnal change in leptin secretion (Sinha *et al.*, 1996b).

Table 4.1: Baseline values of physical and biochemical characteristics in the control and blockade trials were not significantly different from each other (All P 's>0.05). Data are mean values and standard errors of the means

Subject Characteristics	Control	Propranolol
Body weight (kg)	74.0 \pm 3.4	74.0 \pm 3.4
Sum of skin folds (mm)	154 \pm 16	150 \pm 14
NEFA ($\mu\text{mol}\cdot\text{L}^{-1}$)	349 \pm 48	375 \pm 50
Glucose($\text{mmol}\cdot\text{L}^{-1}$)	4.45 \pm 0.14	4.47 \pm 0.09
Leptin ($\text{ng}\cdot\text{ml}^{-1}$)	8.30 \pm 2.06	7.77 \pm 1.76
Rectal Temperature ($^{\circ}\text{C}$)	37.3 \pm 0.1	37.4 \pm 0.1
Cardiac Frequency ($\text{beats}\cdot\text{min}^{-1}$)	75.1 \pm 1.93	74.9 \pm 1.67

4.3.2 Comparison of Responses to Cold-water Immersion With and Without Propranolol

4.3.2.1 Thermoregulatory Responses

Progressive declines of all body temperatures occurred during the cold-water immersion ($P < 0.05$; Figure 4.2 A, B, and C). There were no significant effects or interactions of propranolol on any of these body temperature responses during the immersion (P 's > 0.05). After one hour of immersion, T_{re} was decreased by -0.79 and -0.89°C , T_{sub} by -8.07 and -8.15°C and T_{msk} by -8.33 and -8.45°C , in the control and blockade trials respectively. The subcutaneous adipose tissue layer forms an insulative function, thereby reducing the amount of heat loss from the body. Both in the control and in the blockade trial, there were significant correlations between the fall in T_{re} during cold-water immersion and the sum on skinfolds ($R^2 = 0.39$ and $R^2 = 0.46$, both P 's < 0.05 ; Figure 4.3). Propranolol did not affect these correlations, as they were not significantly different from each other ($P > 0.05$).

4.3.2.2 Cardiovascular Responses.

As expected, f_c was significantly decreased in the blockade trial ($P < 0.05$). One hour after administration of propranolol, f_c was decreased from $74.9 \text{ b}\cdot\text{min}^{-1}$ at baseline to $67.0 \text{ b}\cdot\text{min}^{-1}$ just prior to immersion ($P < 0.05$). As a result, prior to immersion, f_c was significantly lower in the blockade trial compared to the control trial ($P < 0.05$; Figure 4.4). Propranolol did not affect resting skin and fore arm blood flow, prior to immersion [Skin: $236 (\pm 25)$ versus $232 (\pm 28)$; $P > 0.05$, forearm blood flow: $2.39 (\pm 0.21) \text{ ml}\cdot 100 \text{ ml}^{-1}\cdot\text{min}^{-1}$ versus $2.34 (\pm 0.21) \text{ ml}\cdot 100 \text{ ml}^{-1}\cdot\text{min}^{-1}$; $P > 0.05$, Figure 4.5 A and B].

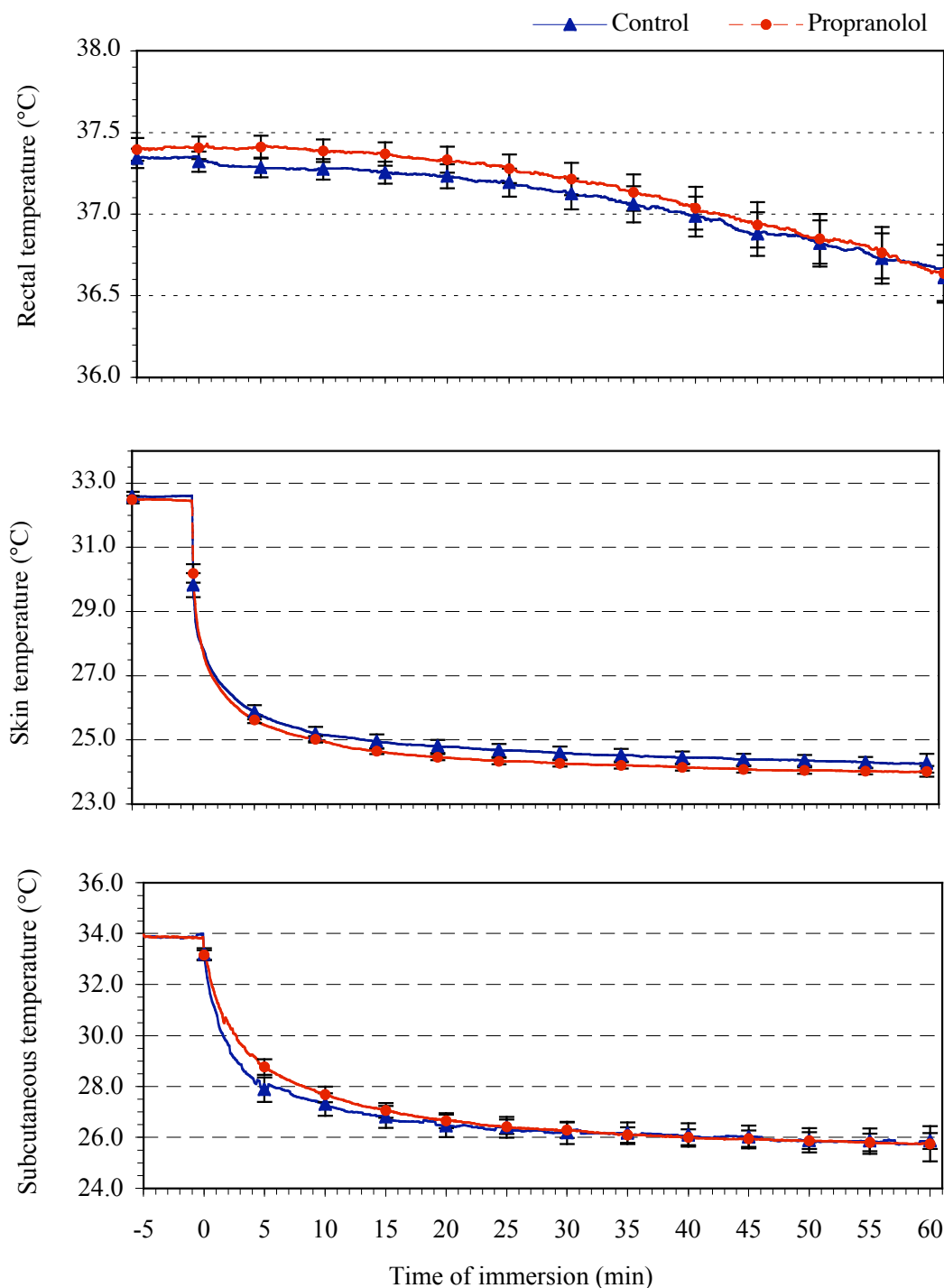


Figure 4.2: Body temperatures during cold water immersion (18°C), without (control) and after administration of 80 mg propranolol A: Rectal temperature B: Mean skin temperature C: Subcutaneous temperature. All body temperatures were significantly affected by the cold-water immersion, but there was no effect or interaction with propranolol (time: P 's<0.05; treatment: P 's>0.05; time*treatment: P 's>0.05). Data are means and standard error of the means.

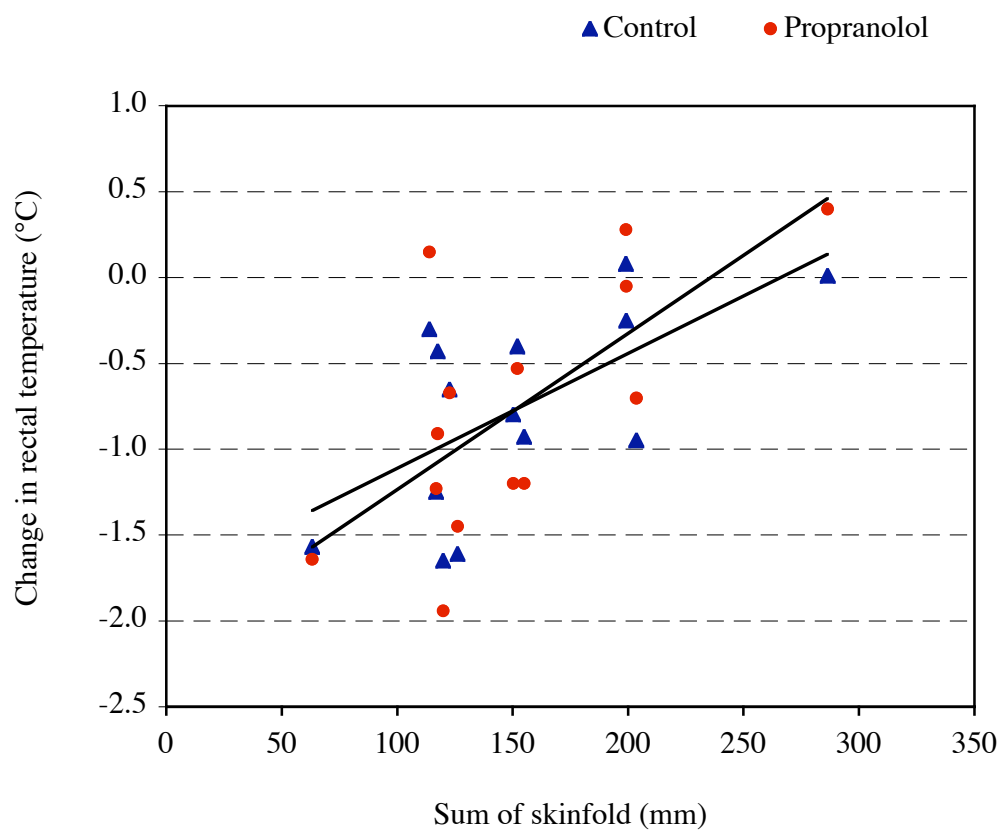


Figure 4.3: Significant correlations between the change in rectal temperature during cold-water immersion (18°C) and the sum of skinfolds (Control: $R^2 = 0.39$; Propranolol: $R^2 = 0.46$, both P 's < 0.05).

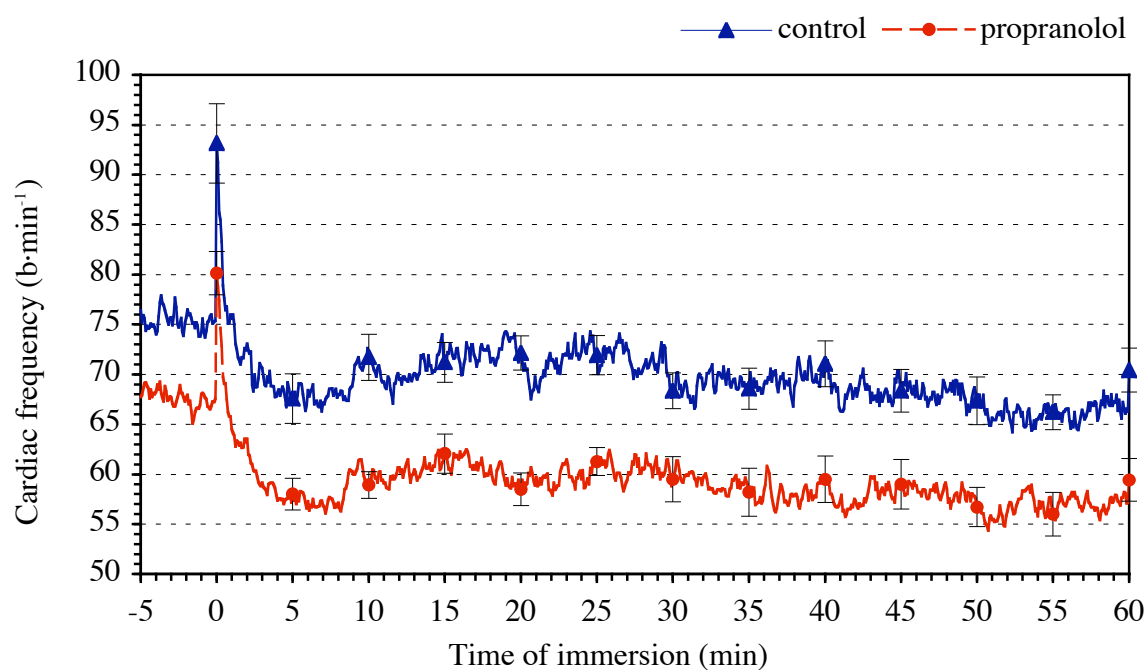


Figure 4.4: Cardiac frequency during cold-water immersion (18°C). 80 mg of propranolol significantly reduced cardiac frequency compared to control. Data are means and standard errors of the means (time: $P < 0.05$, treatment: $P < 0.05$, time*treatment: $P > 0.05$).

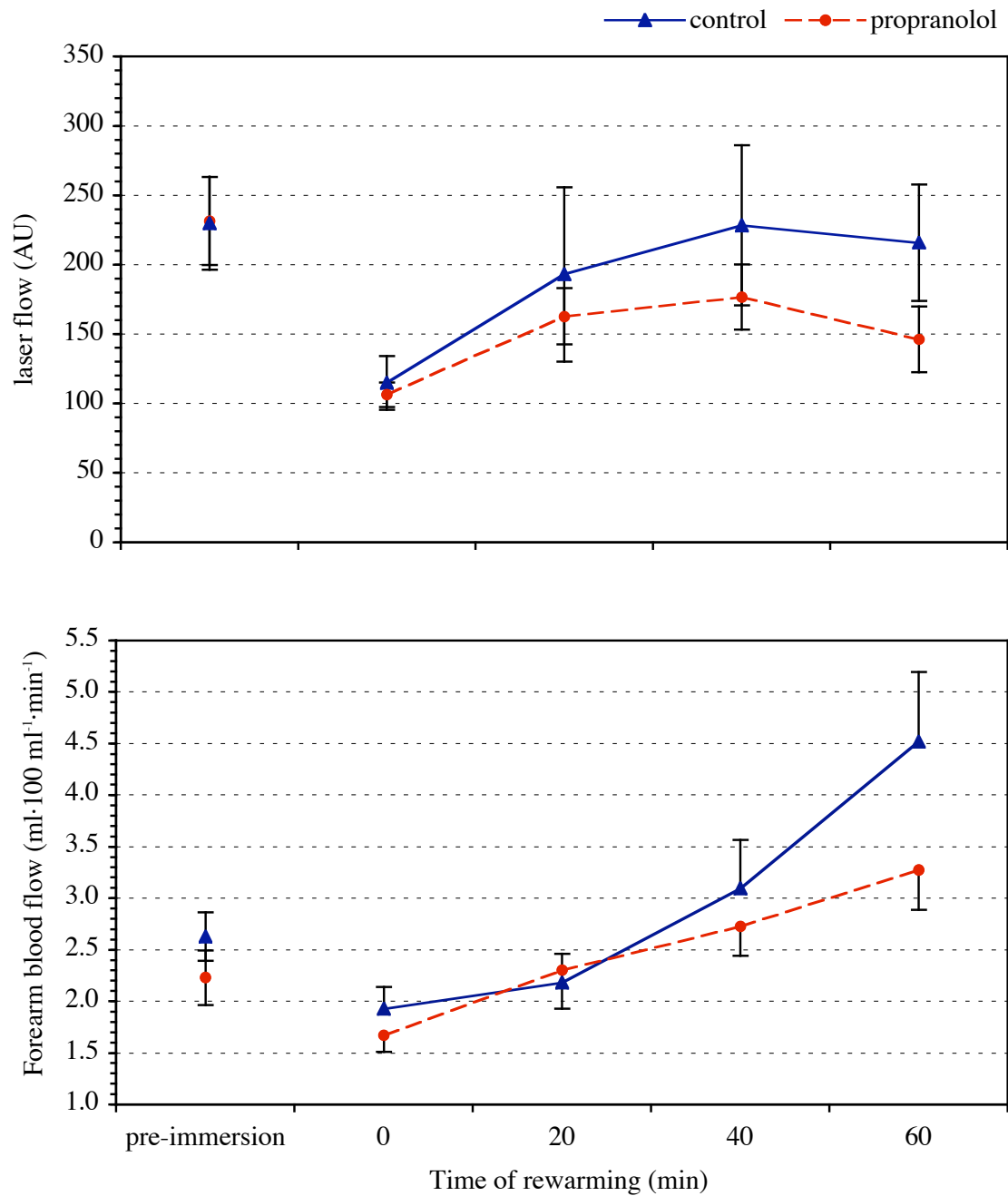


Figure 4.5: Skin blood flow measured by laser doppler flowmetry (A) and forearm blood flow measured by venous occlusion plethmography (B) during cold-water immersion (18°C). **A:** Skin blood flow (Pre-immersion: treatment $P>0.05$; Rewarming: time: $P<0.05$, treatment: $P<0.05$, time*treatment: $P>0.05$). **B:** Forearm blood flow (Pre-immersion: treatment $P>0.05$; Rewarming: time: $P<0.05$, treatment: $P<0.05$, time*treatment: $P>0.05$). Data are means and standard errors of the means.

When comparing f_c between the two trials, a parallel displacement of the cardiac response is observed, with f_c being consistently lower in the propranolol trial than in the control trial. Whilst the water temperature was not profoundly cold, the rapid immersion did induce an initial cold-shock response in both conditions. In the control and blockade trials cardiac frequency increased in absolute terms by $24.1 (\pm 4.3) \text{ b}\cdot\text{min}^{-1}$ and $18.8 (\pm 2.1) \text{ b}\cdot\text{min}^{-1}$ and relatively expressed by $33 (\pm 6) \%$ and $29 (\pm 4) \%$, respectively. Although the values are slightly lower after administration of propranolol, these were not significantly different from each other ($P>0.05$). As the immersion progressed, typical cold-water immersion induced bradycardia was observed in both trials. This was demonstrated by significant decreases in f_c during the first 5 min of immersion, which then remained stable for the remaining duration of immersion.

4.3.2.3 Changes in Circulating Leptin and NEFA, Glucose Concentrations

There was no significant difference between the control and the blockade trials in plasma leptin concentration prior to immersion. Both raw and log-transformed leptin values indicated significant effects of cold-water immersion on plasma leptin concentration and significant interactions of propranolol on the cold-induced decreases in circulation leptin. During the cold-water immersion plasma leptin concentration decreased in both trials. However, in the blockade trial the onset of the decrease was delayed by propranolol, as indicated by a significant interaction (time*treatment $P<0.05$; Figure 4.6 A). In both conditions, plasma leptin significantly decreased during cold-water immersion (both P 's <0.05). At the end of the cold water immersion circulating leptin concentration was decreased to a similar extent in both trials ($P>0.05$).

As a result of the blockade of the beta-adrenergic receptors by propranolol, prior to immersion circulating NEFA tended to be lower compared to the control condition although this difference was not statistically significant ($P=0.07$; Figure 4.6 B). The inhibitory effect of propranolol on NEFA release became apparent during the cold-water immersion. During the immersion, cold-induced lipolysis was almost completely abolished by propranolol (time*treatment $P<0.05$). However, in both conditions, cold-water immersion had a significant effect on circulating NEFA (both P 's <0.05). After one hour of immersion, plasma NEFA concentrations were raised on average by

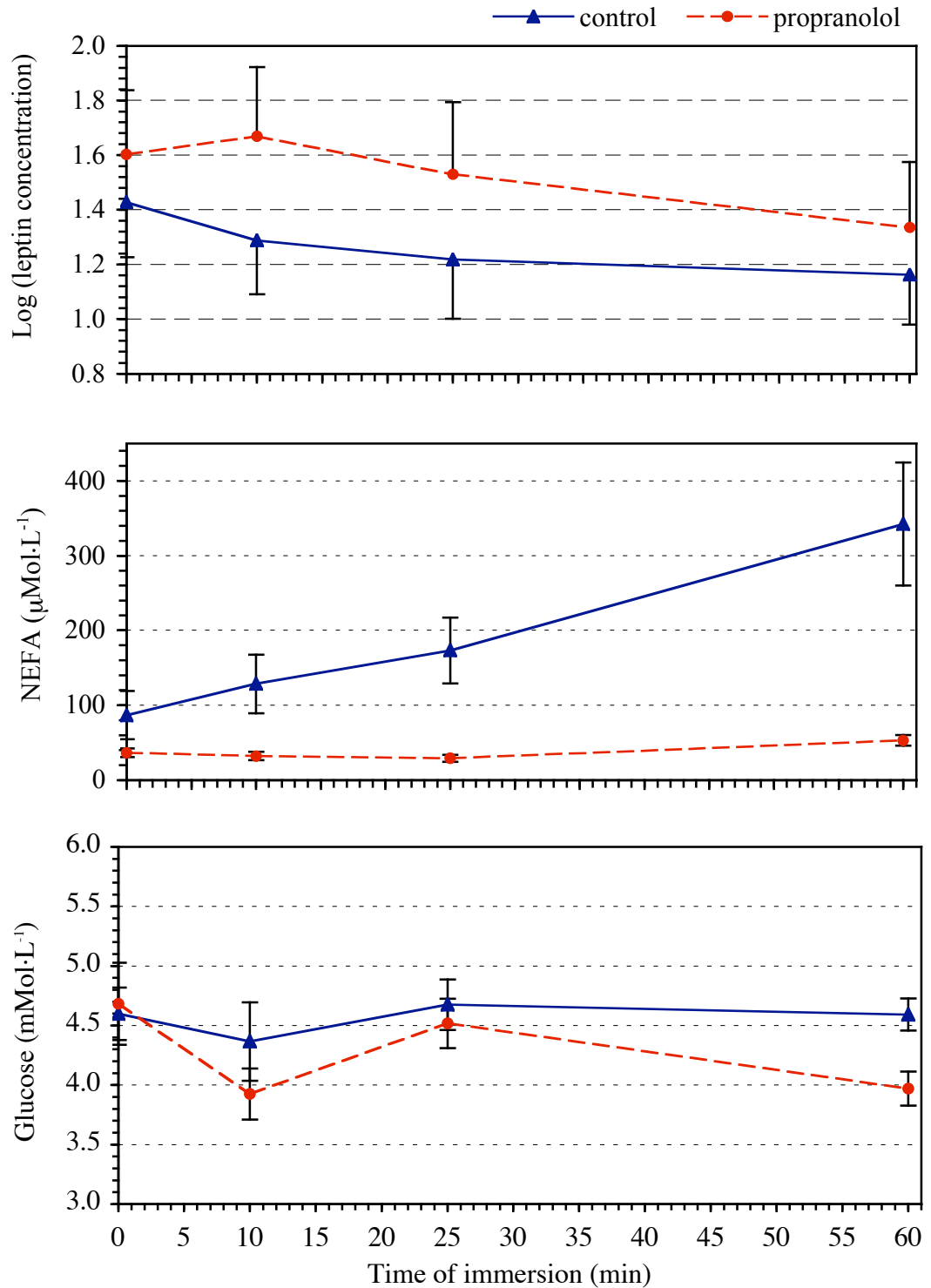


Figure 4.6: Log transformed leptin, Non-esterified fatty acids (NEFA) and glucose concentrations during cold-water immersions (18°C), without (control) and after administration of propranolol. **A:** Leptin (time: $P<0.05$; treatment: $P>0.05$; time*treatment: $P<0.05$). **B:** NEFA (time: $P<0.05$; treatment: $P<0.05$; time*treatment: $P<0.05$). **C:** Glucose: (time: $P>0.05$; treatment: $P>0.05$; time*treatment: $P>0.05$) Data are means and standard errors of the means.

5 fold in the control trials, while in the blockade trials there was only a 1.5 fold increase ($P<0.05$).

Prior to immersion there was no significant difference in mean plasma glucose concentration in the control trials compared to the blockade study [$4.80 (\pm 0.37)$ versus $5.14 (\pm 0.55)$ mMol·L⁻¹]. The response of circulating glucose levels to cold-water immersion did not differ between the two conditions (time*treatment $P>0.05$). Overall there was a significant effect of cold-water immersion on circulating glucose concentration ($P<0.05$; Figure 4.6 C). Plasma glucose concentration fluctuated during immersion, being decreased after 10 minutes of immersion, slightly increased after 25 minutes and decreased after 60 minutes. However, when comparing glucose concentrations at the end of the immersions, there was a non-significant trend for decreased circulating glucose by propranolol after cold-water immersion compared to control ($P=0.06$).

4.3.3 Comparison of Responses to Rewarming after Cold-water Immersion With and Without Propranolol

4.3.3.1 Thermoregulatory Responses

Propranolol did not affect the rewarming responses on either T_{msk} , T_{sub} T_{re} (P 's time*treatment $P>0.05$; Figure 4.7). At the onset of rewarming, T_{msk} and T_{sub} immediately started to increase ($P<0.05$). While T_{msk} reached pre-immersion values within 15 minutes, T_{sub} returned to pre-immersion values after 30 minutes of rewarming (Figure 4.7 B and C). In contrast, T_{re} continued to decrease upon rewarming, this phenomenon is known as the afterdrop ($P<0.05$; Figure 4.7 A). The nadir of the afterdrop was not reached till at least 50 min of rewarming. The magnitude of afterdrop was significantly greater in the propranolol condition compared to the control condition ($P<0.05$; Figure 4.8).

4.3.3.2 Cardiovascular Responses

The cardiac response to rewarming was not affected by beta-adrenergic blockade by propranolol (time*treatment $P>0.05$). Rewarming resulted in significant

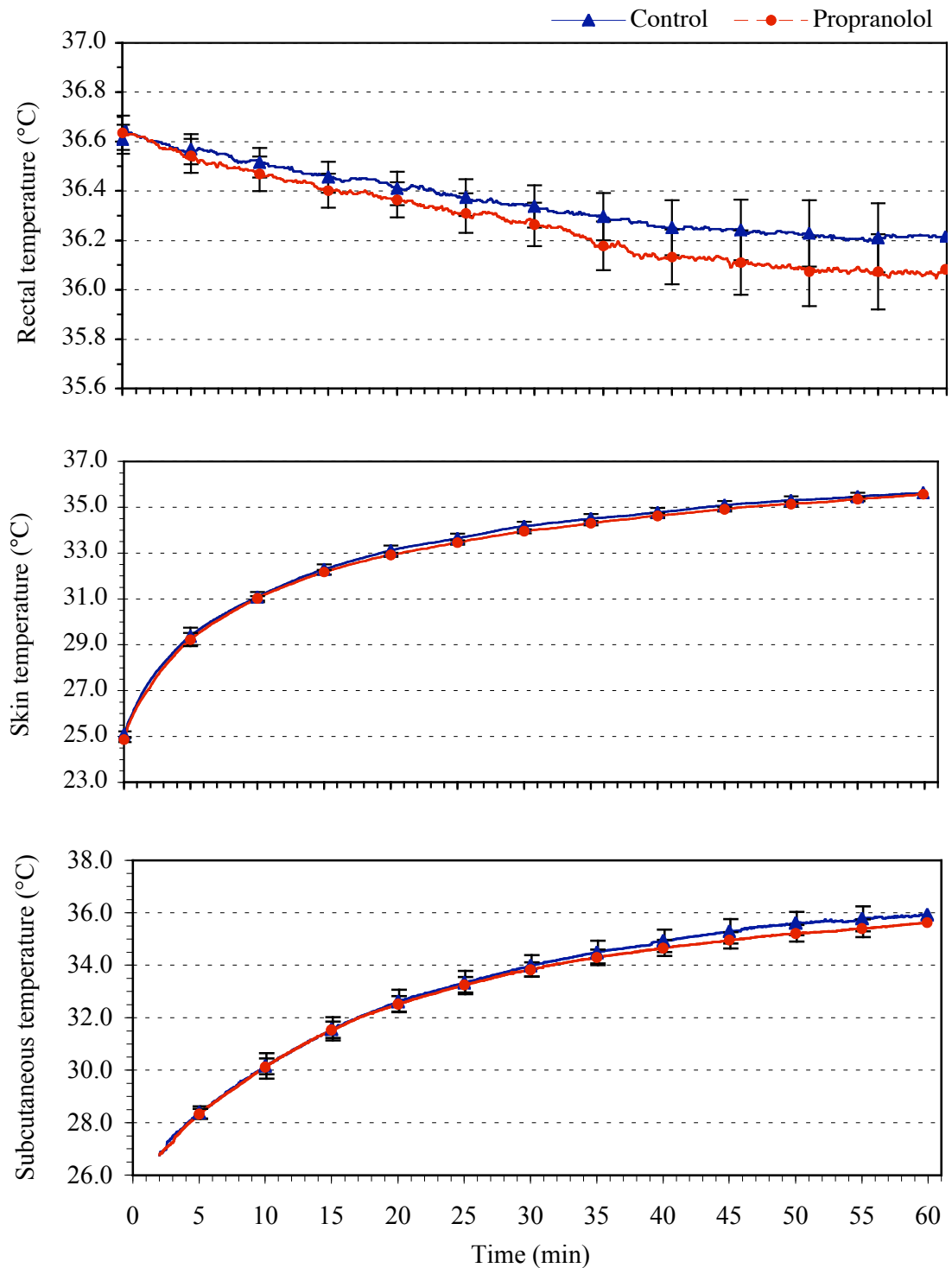


Figure 4.7: Body temperatures during rewarming in a heated climate chamber (38.2°C) after cold water immersion (18°C). **A** Rectal temperature: (time: $P < 0.05$; treatment: $P > 0.05$; time*treatment: $P > 0.05$). **B**: Skin temperature: (time: $P < 0.05$; treatment: $P > 0.05$; time*treatment: $P > 0.05$). **C**: Subcutaneous temperature: (time: $P < 0.05$; treatment: $P > 0.05$; time*treatment: $P > 0.05$). Data are means and standard errors of the means.

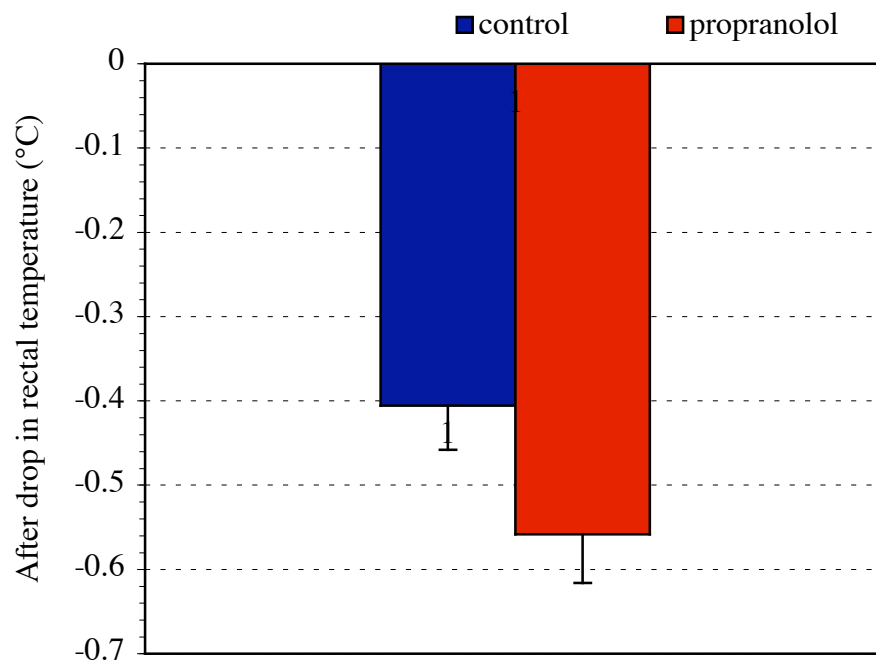


Figure 4.8: The 'afterdrop' in rectal temperature during rewarming in a heated climate chamber (38.2°C) after 60 minutes of cold-water immersion (18°C), without (control) and after administration of 80 mg propranolol (treatment: $P<0.05$). Data are means and standard errors of the means.

increases in f_c in both conditions ($P<0.05$). However during the 60 min rewarming period f_c remained significantly lower in the blockade trials than in the control trials ($P<0.05$; Figure 4.9).

Immediately after the 60 min of cold-water immersion in the heated climate chamber, both skin and forearm blood flows were significantly lower compared to their pre-immersion values ($P<0.05$, Figure 4.5 A and B). Rewarming resulted in significant increases during the rewarming period of both blood flows in both conditions ($P<0.05$). These increases in both skin and forearm blood flows were however suppressed in the blockade trial (treatment $P<0.05$).

4.3.3.3 Circulating Leptin, NEFA and Glucose Concentrations During Rewarming

Plasma leptin concentrations decreased to a similar level during the cold-water immersion, and propranolol did not affect the rewarming dynamics of plasma leptin concentration (time*treatment $P>0.05$; Figure 4.10 A). Rewarming had no significant effect on plasma leptin concentration ($P>0.05$), although it seems there was a slow, but non-significant trend of plasma leptin to increase during this period.

Similarly, there was no effect of propranolol on circulating NEFA responses during the rewarming period (time*treatment $P>0.05$). However, NEFA concentrations remained suppressed by propranolol throughout the rewarming period ($P<0.05$; Figure 4.10 B) and there was no significant increase of NEFA concentration over time in both conditions ($P>0.05$). It could be suggested that the suppression of NEFA release by propranolol tended to wear off during the latter part of the rewarming period. This is supported by the observation that at the end of the immersion, in the propranolol trials mean NEFA concentration was only 25% of NEFA concentration in the control trial, while at the end of rewarming this proportion had increased to 40% ($P<0.05$).

At the start of rewarming glucose concentrations was slightly lower in the propranolol condition. However during the rewarming period there was no significant interaction or effects of either time or treatment on plasma glucose concentration (all

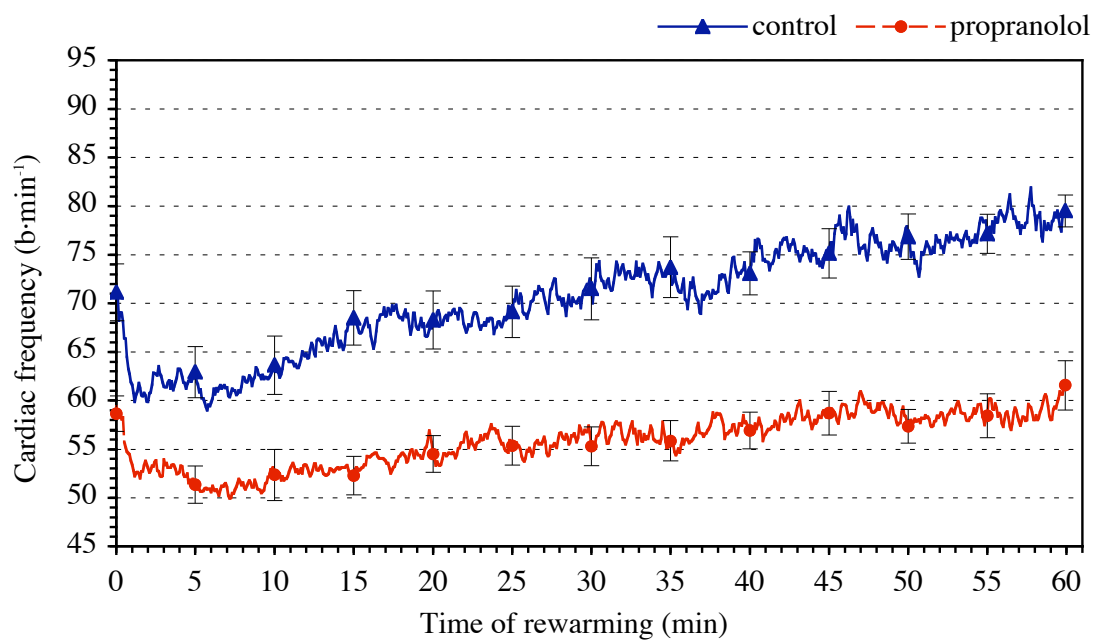


Figure 4.9: Cardiac frequency during rewarming in a heated climate chamber (38.2°C) after cold water (18°C) immersion. (time: $P < 0.05$; treatment: $P < 0.05$; time*treatment: $P > 0.05$). Data are means and standard errors of the means.

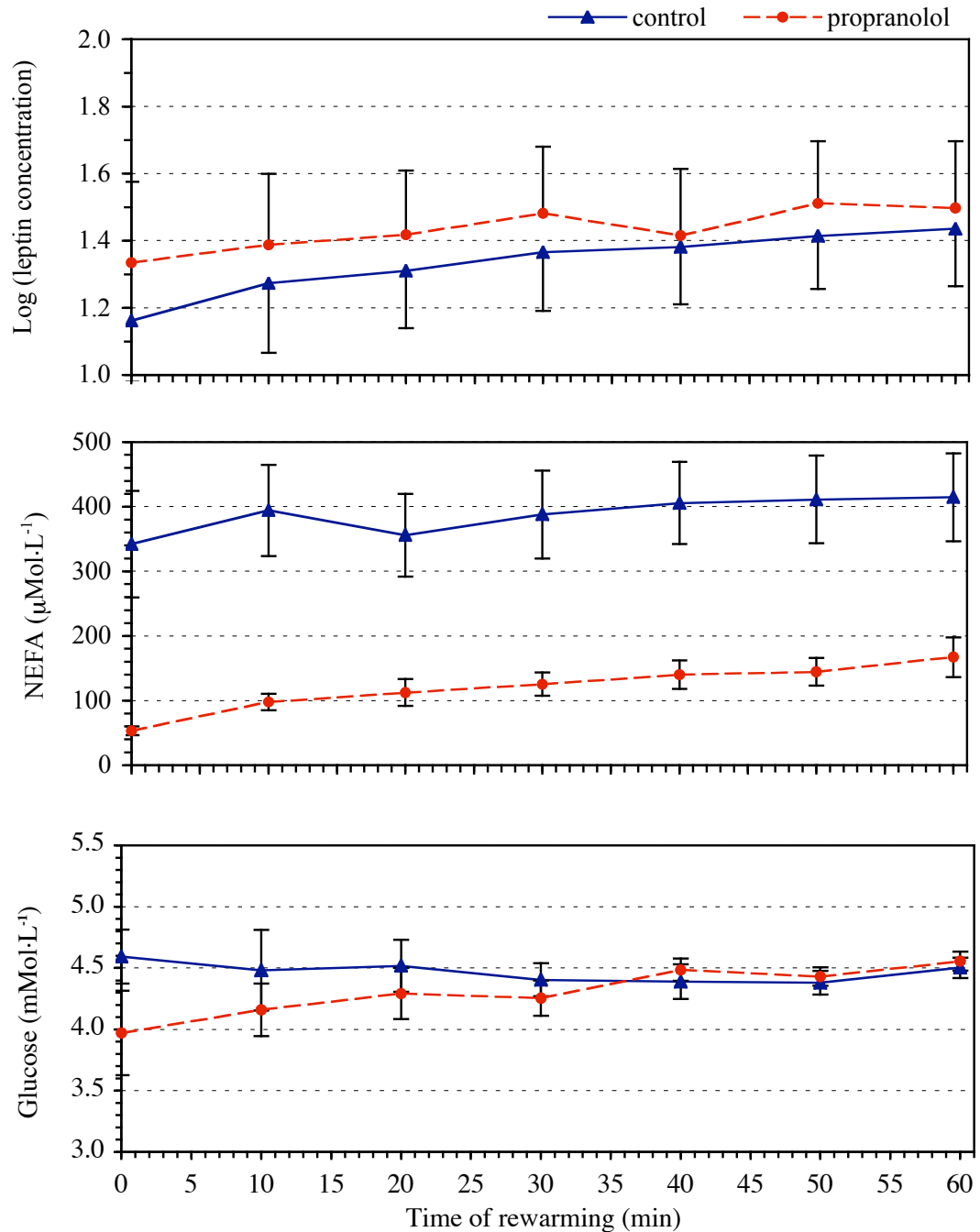


Figure 4.10: Log transformed leptin, Non-esterified fatty acids (NEFA) and glucose concentrations during rewarming in a heated climate chamber (38.2°C) after cold-water immersions (18°C), without (control) and after administration of 80 mg propranolol. A Leptin: (time: $P>0.05$; treatment: $P>0.05$; time*treatment: $P>0.05$). B NEFA: (time: $P<0.05$; treatment: $P<0.05$; time*treatment: $P>0.05$). C Glucose (time: $P>0.05$; treatment: $P>0.05$; time*treatment: $P>0.05$). Data are means and standard errors of the means.

P 's >0.05 ; Figure 4.10 C). During the rewarming the concentrations came together and seemed to be quite similar in the second half of the rewarming period.

4.4 DISCUSSION

4.4.1 The Effect of Beta-adrenergic Blockade on Circulation Leptin During Cold Exposure

To our knowledge this is the first human study, which investigated the effects of beta-adrenergic blockade by propranolol on cold-induced decreases in plasma leptin concentration. This study has demonstrated that beta adrenergic blockade by propranolol, which significantly reduced cold-induced NEFA release from adipose tissue from 295% to 45% ($P<0.05$), did not prevent the cold-induced decrease in plasma leptin (-18.6% and -16.7%, $P>0.05$). These results indicate that the two secretion pathways of leptin and NEFA in human adipocytes are independently controlled.

Cold-induced decreases in both circulating leptin and *ob* gene expression have previously been demonstrated (Trayhurn *et al.*, 1995; Hardie *et al.*, 1996b; Ricci *et al.*, 2000; Asakuma *et al.*, 2003) and are often considered to result from increased sympathetic activation during cold-exposure. Furthermore, beta-adrenergic receptor stimulation induces reduction in plasma leptin concentration as demonstrated in several human *in vivo* and *in vitro* studies (Pinkney *et al.*, 1998; Ricci and Fried, 1999; Scriba *et al.*, 2000). Although these results indeed indicate beta-adrenergic involvement in leptin regulation, they do not confirm a causal relationship between cold-induced reductions in plasma leptin and increased sympathetic activation in the cold.

Ricci *et al.* (2000) conducted, to our best knowledge, the only other human study to the effects of cold exposure on circulating leptin. In this study, after 30, 60 and 90 min of cold-air exposure, plasma leptin concentration had decreased by -11%, -14% and -23%, respectively in healthy young women. This slightly smaller effect after 60 min of cold exposure compared to the current study (-14% compared to -18.6%) can likely be explained by the different intensity of applied cold-stimuli in the two studies, since the conductivity of water is 25 times greater than that of air. The decrease in oesophageal temperature observed by Ricci *et al.* of 0.2°C (from 37.2 to 37.0°C) is

indeed smaller than the 0.7°C (from 37.3 to 36.6°C) decrease in rectal temperature in current investigation. Although, the two measured indices of core temperature, oesophageal and rectal, are not 100% comparable, this significant difference in core temperature response seems to support differences of the extent of the applied thermal stress. Ricci *et al.* reported that cold-induced decreases in plasma leptin were accompanied by increases in plasma norepinephrine (263%, 323% and 454%) and glycerol (38%, 77% and 110%). Similarly to authors of several rodent studies, Ricci *et al.* suggest that the cold induced decrease in leptin result from the increased sympathetic activation.

To our best knowledge, there is only one other study that used beta-adrenergic antagonists to investigate sympathetic regulation of cold-induced changes in leptin physiology. Evans *et al.* concluded that the combination of both propranolol (non selective beta adrenergic agonist) and SR 59230A (beta₃ selective agonist) was required for cold-induced modifications of serum leptin and *ob* mRNA expression in brown and white adipose tissue in mice (Evans *et al.*, 1999). However, as previously discussed it may be argued that different ways of statistical analyses of their data would indicate that the beta-adrenergic blockades did not prevent cold-induced in leptin physiology. Furthermore, it has been demonstrated that the beta-adrenergic regulation of leptin differs between humans and rodents (Scriba *et al.*, 2000).

In the current study the applied level of blockade did not prevent the fall in leptin concentration at the end of immersion, however the decrease in circulating leptin was delayed by propranolol. Upon immersion, the sudden stimulation of peripheral cold receptors in the skin, results in an immediate sympathetic activation accompanied by a rapid increase in circulating norepinephrine (Johnson *et al.*, 1977). It therefore could be suggested that propranolol did indeed block cold-induced decreases in plasma leptin concentration due to initial large increase of sympathetic outflow. However, Johnson *et al.* demonstrated that norepinephrine remains elevated when cold exposure continues. Despite the fact that propranolol effectively suppressed cold-induced NEFA release throughout the complete duration of the immersion, it did not prevent the decrease in plasma leptin concentration as the immersion continued. Local and direct effects of temperature are often not considered as of physiological significance with respect to cold-induced effects on leptin physiology. However, the *in vitro* results presented and

discussed in the previous chapter (Chapter 2 and 3) and of Peino *et al.* demonstrated leptin secretion rate is reduced at lower adipose tissue temperature (Peino *et al.*, 2000). Additionally, the mathematical model described in Chapter 2 supports an important contribution of local, direct effect of reduced adipose tissue temperature during cold-exposure. These data suggest that, while the initial cold immersion response may be due to sympathetic outflow, as cold exposure continues, direct local effects dominate the circulating leptin response.

4.4.2 The Effect of Beta-adrenergic Blockade on Thermoregulatory Responses

Due to the great conductivity of water, significant body heat loss occurred from the periphery to the core, demonstrated by progressive declines of all body temperatures during the cold-water immersion. The responses of any of the measured body temperatures to cold-water immersion were not affected by propranolol. These results differ from the results of Simeckova *et al.* (2000), who demonstrated greater decreases in T_{re} during cold-water immersion after administration of propranolol when compared to control. However, the water temperature in that study was 12°C compared to 18°C in the current investigation, therefore the cold stress that was applied in the study of Simeckova *et al.* was greater. It has been suggested that a certain heat debt is required for activation of heat production independent of muscular activity. This non-shivering thermogenesis, suggested to be the result of norepinephrine mediated increased NEFA release and subsequent fat metabolism, is activated when T_{re} has decreased by at least 0.5°C (Jessen, 1980). In the present study, such decreases in T_{re} were reached only in the last 15 minutes of the immersion. However, while skin temperature and subcutaneous temperature started to increase almost immediately upon entering the heated chamber, while rectal temperature continued to decrease. Propranolol did not affect the rewarming dynamics of any of the body temperatures, however the magnitude of the afterdrop observed in T_{re} was significantly increased in the blockade trial. The afterdrop has often been ascribed to changes in peripheral blood flow. Skin and forearm blood flow significantly increased during rewarming in both conditions ($P < 0.05$). However, as a result of blocking of the beta-adrenergic receptor mediated vasodilation, both skin (-32%) and forearm blood flow (-27%) were lower in the propranolol condition. Although contribution of the circulatory and conductive components to the afterdrop phenomenon cannot be excluded, they do not explain the observed difference

in afterdrop between the two conditions. While circulating levels of NEFA and glucose are not accurate metabolic indicators, as both are the result of total production and (re)uptake by the whole body, we can deduce certain information from the comparison of circulating levels over time and between the control and blockade trial. Reduced availability of NEFA did not impair thermoregulatory function during cold water immersion, however it may clarify the increased after drop during beta-adrenergic blockade due to a reduced thermogenic capacity during rewarming.

4.4.3 Conclusions

This study demonstrates that not only increased sympathetic outflow during cold exposure contributes to cold-induced decreases in leptin physiology. While the applied level of beta-adrenergic blockade was sufficient to almost completely suppress cold-induced NEFA release, it did not prevent plasma leptin concentration to decrease. From these results and together with the *in vitro* results from Chapter 3, it can be concluded that local and direct effects significantly contribute to the cold-induced effects on leptin physiology. Cardiac frequency was lowered and parallel displaced by propranolol. No effects of beta-adrenergic blockade on thermoregulatory responses were demonstrated during immersion in 18°C water and subsequent rewarming. However, during the latter period propranolol increased the afterdrop, which was probably due to reduced thermogenic response due to suppressed NEFA release.

CHAPTER 5: CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE RESEARCH

While obesity is in the media often portrayed as a new disease and suggested to be the result of a modern society with an established fast food industry it was already noted in 1936 that ‘obesity is one of the most common diseases’ (Du Bois, 1936). Maintenance of body weight is dependent on accurate control of energy balance. Accordingly changes in body weight are simply the result of energy imbalance *i.e.* when energy expenditure does not equal energy intake. Cross sectional data of various indigenous male groups over the world demonstrate significant relationships between climate (temperature) and body mass variation indicating that environmental temperature affects mechanisms involved in body weight regulation (Roberts, 1978). Experimental human studies of the effects of environmental temperature have been primarily been concerned with the metabolic or thermal responses (Martineau and Jacobs, 1989; Prisby *et al.*, 1999; Sramek *et al.*, 2000), with little consideration for energy intake. In contrast, abundant animal data demonstrate cold-induced effects on both food intake and energy expenditure (Morrison, 1981; Collier *et al.*, 1989). However, underlying mechanisms by which temperature may affect body composition have hardly been addressed or investigated.

Leptin, a hormone secreted by adipose tissue in direct proportion to the amount of body fat stored, is considered an afferent signal to the CNS providing information on energy storage. Leptin has effects on both sides of the energy balance equation as it affects specific receptors in the brain involved both energy intake and expenditure (Jequier and Tappy, 1999; Woods *et al.*, 2000; Cupples, 2005). Short-term changes in plasma leptin and *ob* gene expression independently of alteration in adiposity occur during fasting and cold exposure. These are often considered to result from increased sympathetic activity during the cold, while local temperature effects on leptin physiology are relatively unexplored (Trayhurn *et al.*, 1995; Hardie *et al.*, 1996b; Ricci *et al.*, 2000; Asakuma *et al.*, 2003). Furthermore, while it has been demonstrated that cold-induced hyperphagia is accompanied by decreased circulating leptin concentration (Bing *et al.*, 1998) a possible role for leptin in body composition regulation in response to environmental temperature changes has not previously been addressed. Hence, the aim of this investigation was to investigate possible interactions between environmental

temperature and the regulation of human body composition. Evidence has been sought for direct temperature effects on leptin as a possible mechanism for temperature effects on energy balance regulation in humans.

The purpose of the first study was to identify the effects of single and repeated cold-water immersions on circulating leptin concentration in humans. During cold-water immersion there was an acute decrease in plasma leptin concentration, similar to earlier demonstrated effects during cold-air exposure in both human and rodents. Also a significant relationship between plasma leptin concentration and the fall in T_{re} during cold-water immersion was found which may be indicative of a protective role of leptin to reduce the fall in T_{re} during repeated cold exposure. Finally, this study demonstrated for the first time, a longer-term, positive effect of repeated cold exposure on plasma leptin concentration.

The second study focused upon direct and local temperature effects on leptin secretion by human adipose tissue fragments. The principle observation was that at lower incubation temperature leptin secretion is reduced. Using a mathematical model of leptin dynamics incorporating these *in vitro* results, the *in vivo* results from the first study could be explained by local effects of temperature on leptin secretion rate. Thus, the local effects of reduced subcutaneous adipose tissue temperature may be a more important contributor to the acute effects observed *in vivo*, than the sympathetically mediated suppression of leptin secretion.

Finally, the purpose of the third study was to determine whether cold-induced decrease in plasma leptin concentration was prevented by the beta-adrenergic antagonist, propranolol, and to investigate the effects of beta-adrenergic blockade on the physiological responses of cold-water immersion and subsequent rewarming. The beta-adrenergic blockade prevented cold-induced lipolysis, however plasma leptin concentration at the end of 60 min of immersion was decreased to a similar extent in both conditions. Cardiac frequency was significantly reduced during cold-water immersion. Thermoregulatory responses during cold-water immersion were not affected by the beta-adrenergic blockade. There was no effect of the beta-adrenergic blockade on circulating leptin during the rewarming period. The afterdrop in rectal temperature was

increased in the blockade trial while cardiac frequency remained suppressed demonstrated by a parallel displacement between the control and blockade trial.

5.1 CONCLUSIONS

The presented investigations provide several lines of evidence for a role of leptin in energy balance regulation in response to environmental temperature changes. First of all, cold-water immersion resulted in an acute and significant reduction in plasma leptin concentration. Cold-induced decrease in human plasma leptin concentration is not an unique finding as while this work was in progress another human study also reported cold-induced changes in circulating leptin concentration (Ricci *et al.*, 2000). The current investigation however was the first to demonstrate that repeated cold-water immersions resulted in an increased leptin concentration. Local temperature effects on leptin secretion *in vitro* have been previously been demonstrated (Peino *et al.*, 2000). However, the implications of the magnitude of the *in vitro* response to temperature change for the cold-induced suppression of circulating leptin *in vivo* has not previously been addressed. The mathematical model together with the beta-adrenergic blockade study together provide strong evidence that local effects of reduced subcutaneous adipose tissue temperature may be a more important contributor to the acute effects observed *in vivo*, than sympathetically mediated suppression of leptin secretion.

Cold-induced hyperphagia is accompanied by decreases in leptin concentration and *ob* gene expression (Bing *et al.*, 1998), however the current literature tends to overlook the possibility for a role of leptin in underlying temperature driven effects on energy balance. An interesting example is a review published in 2002 (after the discovery of leptin) on temporal organization of ingestive behaviour (Strubbe and van Dijk, 2002). In one section of the paper, the authors review a number of studies (all conducted prior to the discovery of leptin) on ingestive behaviour signals of the body associated with metabolism during cold-acclimatisation. The studies indicate that cold-induced increases in energy expenditure are compensated by an increased food intake and provide evidence that this is mainly achieved by an increase in meals size rather than by frequency. The authors conclude that these results indicate a simple regulatory feedback loop of which the mechanism is unclear. Further along in the same paper results of the authors' own experiments are discussed demonstrating that the effects of

leptin on food intake are to reduce the size of a meal rather than affecting the meal frequency. While at the time of publication (2002), the effects of cold exposure on leptin concentration were well established and this paper discusses both the effects of cold on food intake and leptin of food intake, the involvement of leptin in increased food intake in the cold was not considered (Strubbe and van Dijk, 2002).

Another example is a recent human study that investigated the responses of exercise in cold-water compared to neutral water in healthy subjects (White *et al.*, 2005). As previously demonstrated exercise in cold-water resulted in higher energy expenditure and lower tympanic temperature compared the neutral condition. This study also investigated energy intake in the hour following the exercise period. Energy intake after exercise in cold-water was significantly higher than compared to the neutral water trial. Furthermore, the energy intake was twice the amount of energy expended during exercise in cold-water, resulting in an overall positive energy balance at the end of the cold-water trial. While circulating concentrations of leptin were not determined in this study, it could be suggested that reduced levels of circulating leptin may be underlying the increased energy intake after exercise in cold-water.

In other studies, effects of temperature on leptin seem apparent in the data, but are overlooked by the authors and are therefore not further discussed. There are two studies from a same lab group which investigated the effects of peripheral administration of leptin after a high and low fat diet (Bowen *et al.*, 2003; Haltiner *et al.*, 2004). The two studies were conducted at different ambient temperatures (23°C and 27°C) and comparing leptin concentrations in the control animals, it is apparent that plasma leptin concentration was significantly lower in the study conducted at 23°C. Furthermore, from the presented data it can also be suggested that at 23°C plasma leptin concentration was not affected by dietary intake, while at 27°C low fat diet increased please leptin concentration. This may suggest that at a lower temperature leptin secretion is not only suppressed, but also the mechanism by which diet influences leptin regulation seems to be less responsive at the lower ambient temperature.

It should be considered that leptin is not the only possible candidate involved in the regulation of energy balance in response to environmental temperature and that other substances should also be considered. However while chronic administration of

leptin has a long term effect on food intake and body weight, other typical satiety hormones such as CCK and GLP-1 only have short-term suppressive effect on food intake but do not seem to be involved the long term regulation of body weight (Cupples, 2005). Furthermore, a feedback mechanism as currently proposed requires that the signal originate from the energy depot itself thus an adipocytes derived product. While there are more adipocyte products than leptin, these seem not to be affected by temperature changes. For example, no significant effects of cold exposure were found on circulating adiponectin nor on either BAT and WAT *mRNA* levels of resistin and adiponectin (Puerta *et al.*, 2002).

5.2 FUTURE RESEACH

Further investigation is needed to determine further implications of temperature effects on human energy balance. A scientific understanding of homeostatic mechanisms in response to environmental temperature may be more important in human than in most other mammals because human behavioural responses to temperature (clothing, heating etc) may interact the homeostatic mechanisms such that energy balance is not achieved with consequential effect on body composition.

Cold-induced reductions in leptin physiology are now well established but future studies are required to further differentiate and quantify the centrally mediated and local temperature effects on leptin physiology during the course of cold exposure. While the increased sympathetic outflow may be the main mechanism at the initial phase of cold-exposure, this investigation has demonstrated that direct, local effects become of greater importance when cold-exposure continues.

In terms of mechanism, future studies should attempt to measure *ob* gene expression preferably at various points in time during the cold-exposure. This will enable determination of the extent of pre- or/and post-transcriptional effects as the underlying mechanism. Recently it has been demonstrated that the inhibitory effect of isoproterenol on leptin secretion from human subcutaneous adipose tissue are post-transcriptional (Ricci *et al.*, 2005). The mechanism for direct, local effects temperature on leptin release has to our knowledge not yet been investigated in human subcutaneous adipose tissue. The study that investigated temperature effects on leptin secretion by

human omental adipose tissue (Peino *et al.*, 2000) demonstrated no direct temperature effects on *ob* gene expression. These results seem to suggest that both centrally as locally mediated effects of temperature occur at a post-transcriptional level. However, future *in vivo* studies would benefit from collection of human subcutaneous adipose tissue samples before and after cold-exposure. Due to the invasive character of subcutaneous adipose tissue sampling, cold air would be a more appropriate medium than cold-water. Additionally, local measurement of leptin in the interstitial fluid subcutaneous adipose tissue depot by microdialysis, a relatively new *in vivo* sampling technique, could provide better insight into the mechanisms. Also this technique would be more appropriate and feasible in a cold-air experiment than cold-water immersion trials.

Ultimately, future studies should include measurements of both energy expenditure and energy intake to assess the overall effects of temperature on energy balance. Energy expenditure during and after cold exposure can be determined by respiratory gas-exchange measurements and *ad libitum* food intake following cold-exposure by providing free access to a varied assortment of food items. These measurements need to be obtained in parallel with determination of circulating leptin concentrations.

CHAPTER 6: REFERENCES

- Arner, P. (1995). Techniques for the measurement of white adipose tissue metabolism: a practical guide. *International Journal of Obesity and Related Metabolic Disorders* 19: 435-442.
- Arner, P., Arner, O., and Ostman, J. (1973). The effect of local anaesthetic agents on lipolysis by human adipose tissue. *Life Sciences* 13: 161-169.
- Asakuma, S., Morishita, H., Sugino, T., Kurose, Y., Kobayashi, S., and Terashima, Y. (2003). Circulating leptin response to feeding and exogenous infusion of insulin in sheep exposed to thermoneutral and cold environments. *Comparative Biochemistry and Physiology. Part A, Molecular and Integrative Physiology* 134: 329-335.
- Atterhog, J.H., Carlens, P., Granberg, P.O., and Wallenberg, L.R. (1975). Cardiovascular and renal responses to acute cold exposure in water-loaded man. *Scandinavian Journal of Clinical and Laboratory Investigation* 35: 311-317.
- Bartness, T.J. and Bamshad, M. (1998). Innervation of mammalian white adipose tissue: implications for the regulation of total body fat. *American Journal of Physiology*.
- Bergmann, C. (1847). Ueber die Verhältnisse der wärmeökonomie der Thiere zu ihrer Grösse. In: *Göttinger Studien*. Göttingen, p. 595-708.
- Bing, C., Frankish, H.M., Pickavance, L., Wang, Q., Hopkins, D.F., Stock, M.J., and Williams, G. (1998). Hyperphagia in cold-exposed rats is accompanied by decreased plasma leptin but unchanged hypothalamic NPY. *American Journal of Physiology* 274: R62-R68.
- Boden, G., Chen, X., Mozzoli, M., and Ryan, I. (1996). Effect of fasting on serum leptin in normal human subjects. *Journal of Clinical Endocrinology and Metabolism* 81: 3419-3423.
- Bowen, H., Mitchell, T.D., and Harris, R.B. (2003). Method of leptin dosing, strain, and group housing influence leptin sensitivity in high-fat-fed weanling mice. *Am J Physiol Regul Integr Comp Physiol* 284: R87-100.
- Bradley, R.L., Cleveland, K.A., and Cheatham, B. (2001). The adipocyte as a secretory organ: mechanisms of vesicle transport and secretory pathways. *Recent Progress in Hormone Research* 56: 329-358.
- Campfield, L.A., Smith, F.J., Guisez, Y., Devos, R., and Burn, P. (1995). Recombinant mouse OB protein: evidence for a peripheral signal linking adiposity and central neural networks [see comments]. *Science* 269: 546-549.

- Caprio, S., Tamborlane, W.V., Silver, D., Robinson, C., Leibel, R., McCarthy, S., Grozman, A., Belous, A., Maggs, D., and Sherwin, R.S. (1996). Hyperleptinemia: an early sign of juvenile obesity. Relations to body fat depots and insulin concentrations. *American Journal of Physiology*.
- Casabiell, X., Pineiro, V., De la Cruz, L.F., Gualillo, O., Folgar, L., Dieguez, C., and Casanueva, F.F. (2000). Dual effect of insulin on in vitro leptin secretion by adipose tissue. *Biochemical and Biophysical Research Communications* 276: 477-482.
- Clement, K., Garner, C., Hager, J., Philippi, A., LeDuc, C., Carey, A., Harris, T.J., Jury, C., Cardon, L.R., Basdevant, A., Demenais, F., Guy, G., North, M., and Froguel, P. (1996). Indication for linkage of the human OB gene region with extreme obesity. *Diabetes* 45: 687-690.
- Coleman, D.L. (1973). Effects of parabiosis of obese with diabetes and normal mice. *Diabetologia* 9: 294-298.
- Collier, G.H., Johnson, D.F., Naveira, J., and Cybulski, K.A. (1989). Ambient temperature and food costs: effects on behavior patterns in rats. *American Journal of Physiology* 257: R1328-1334.
- Considine, R.V., Nyce, M.R., Kolaczynski, J.W., Zhang, P.L., Ohannesian, J.P., Moore, J.H., Jr., Fox, J.W., and Caro, J.F. (1997). Dexamethasone stimulates leptin release from human adipocytes: unexpected inhibition by insulin. *Journal of Cellular Biochemistry* 65: 254-258.
- Coruzzi, P., Biggi, A., Musiari, L., Ravanetti, C., and Novarini, A. (1986). Renal hemodynamics and natriuresis during water immersion in normal humans. *Pflugers Archiv. European Journal of Physiology* 407: 638-642.
- Couillard, C., Mauriege, P., Prud'homme, D., Nadeau, A., Tremblay, A., Bouchard, C., and Despres, J.P. (2002). Plasma leptin response to an epinephrine infusion in lean and obese women. *Obesity Research* 10: 6-13.
- Cupples, W.A. (2005). Physiological regulation of food intake. *Am J Physiol Regul Integr Comp Physiol* 288: R1438-1443.
- Dauncey, M.J. (1981). Influence of mild cold on 24 h energy expenditure, resting metabolism and diet-induced thermogenesis. *British Journal of Nutrition* 45: 257-267.
- Dauncey, M.J. and Ingram, D.L. (1979). Effect of dietary composition and cold exposure on non-shivering thermogenesis in young pigs and its alteration by the beta-blocker propranolol. *British Journal of Nutrition*. 41: 361-370.
- Deng, C., Moinat, M., Curtis, L., Nadakal, A., Preitner, F., Boss, O., Assimacopoulos, J.F., Seydoux, J., and Giacobino, J.P. (1997). Effects of beta-adrenoceptor subtype stimulation on obese gene messenger ribonucleic acid and on leptin

secretion in mouse brown adipocytes differentiated in culture. *Endocrinology* 138: 548-552.

Du Bois, E.F. Basal Metabolism in Health and Disease. edited by Fibiger, L. Philadelphia, 1936.

Epstein, M. (1976). Cardiovascular and renal effects of head-out water immersion in man: application of the model in the assessment of volume homeostasis. *Circulation Research* 39: 619-628.

Evans, B.A., Agar, L., and Summers, R.J. (1999). The role of the sympathetic nervous system in the regulation of leptin synthesis in C57BL/6 mice [published erratum appears in FEBS Lett 1999 May 21;451(2):214]. *FEBS Letters* 444: 149-154.

Fain, J.N., Kanu, A., Bahouth, S.W., Cowan, G.S., Jr., Hiler, M.L., and Leffler, C.W. (2002). Comparison of PGE₂, prostacyclin and leptin release by human adipocytes versus explants of adipose tissue in primary culture. *Prostaglandins Leukotrienes and Essential Fatty Acids* 67: 467-473.

Faulconnier, Y., Delavaud, C., and Chilliard, Y. (2003). Insulin and (or) dexamethasone effects on leptin production and metabolic activities of ovine adipose tissue explants. *Reproduction, Nutrition, Development* 43: 237-250.

Feist, D.D. and White, R.G. Terrestrial mammals in cold. In: *Advances in comparative and environmental physiology*, edited by Wang, L.C.H. Berlin Heidelberg: Springer-Verlag, 1989.

Frank, S.M., Raja, S.N., Bulcao, C., and Goldstein, D.S. (2000). Age-related thermoregulatory differences during core cooling in humans. *American Journal of Physiology - Regulatory Integrative & Comparative Physiology*. 279: R349-354.

Frim, J., Livingstone, S.D., Reed, L.D., Nolan, R.W., and Limmer, R.E. (1990). Body composition and skin temperature variation. *Journal of Applied Physiology* 68: 540-543.

Fruhbeck, G., Jebb, S.A., and Prentice, A.M. (1998). Leptin: physiology and pathophysiology. *Clinical Physiology* 18: 399-419.

Garibotto, G., Russo, R., Franceschini, R., Robaudo, C., Saffioti, S., Sofia, A., Rolandi, E., Deferrari, G., and Barreca, T. (1998). Inter-organ leptin exchange in humans. *Biochemical and Biophysical Research Communications* 247: 504-509.

Gavrilova, O., Leon, L.R., Marcus-Samuels, B., Mason, M.M., Castle, A.L., Refetoff, S., Vinson, C., and Reitman, M.L. (1999). Torpor in mice is induced by both leptin-dependent and -independent mechanisms. *Proceedings of the National Academy of Sciences of the United States of America* 96: 14623-14628.

Giacobino, J.P. (1996). Role of the beta₃-adrenoceptor in the control of leptin expression. *Hormone and Metabolic Research* 28: 633-637.

- Gottschling-Zeller, H., Birgel, M., Scriba, D., Blum, W.F., and Hauner, H. (1999). Depot-specific release of leptin from subcutaneous and omental adipocytes in suspension culture: effect of tumor necrosis factor-alpha and transforming growth factor-beta1. *European Journal of Endocrinology / European Federation of Endocrine Societies* 141: 436-442.
- Halaas, J.L., Gajiwala, K.S., Maffei, M., Cohen, S.L., Chait, B.T., Rabinowitz, D., Lallone, R.L., Burley, S.K., and Friedman, J.M. (1995). Weight-reducing effects of the plasma protein encoded by the obese gene [see comments]. *Science* 269: 543-546.
- Halleux, C.M., Servais, I., Reul, B.A., Detry, R., and Brichard, S.M. (1998a). Multihormonal control of ob gene expression and leptin secretion from cultured human visceral adipose tissue: increased responsiveness to glucocorticoids in obesity. *Journal of Clinical Endocrinology and Metabolism* 83: 902-910.
- Halleux, C.M., Servais, I., Reul, B.A., Detry, R., and Brichard, S.M. (1998b). Multihormonal control of ob gene expression and leptin secretion from cultured human visceral adipose tissue: increased responsiveness to glucocorticoids in obesity. *Journal of Clinical Endocrinology and Metabolism* 83: 902-910.
- Haltiner, A.L., Mitchell, T.D., and Harris, R.B. (2004). Leptin action is modified by an interaction between dietary fat content and ambient temperature. *Am J Physiol Regul Integr Comp Physiol* 287: R1250-1255.
- Haman, F., Peronnet, F., Kenny, G.P., Massicotte, D., Lavoie, C., Scott, C., and Weber, J.M. (2002). Effect of cold exposure on fuel utilization in humans: plasma glucose, muscle glycogen, and lipids. *Journal of Applied Physiology* 93: 77-84.
- Hardie, L.J., Guilhot, N., and Trayhurn, P. (1996a). Regulation of leptin production in cultured mature white adipocytes. *Hormone and Metabolic Research* 28: 685-689.
- Hardie, L.J., Rayner, D.V., Holmes, S., and Trayhurn, P. (1996b). Circulating leptin levels are modulated by fasting, cold exposure and insulin administration in lean but not Zucker (fa/fa) rats as measured by ELISA. *Biochemical and Biophysical Research Communications* 223: 660-665.
- Havel, P.J. (2000). Role of adipose tissue in body-weight regulation: mechanisms regulating leptin production and energy balance. *Proceedings of the Nutrition Society* 59: 359-371.
- Havel, P.J., Kasim-Karakas, S., Mueller, W., Johnson, P.R., Gingerich, R.L., and Stern, J.S. (1996). Relationship of plasma leptin to plasma insulin and adiposity in normal weight and overweight women: effects of dietary fat content and sustained weight loss. *Journal of Clinical Endocrinology and Metabolism* 81: 4406-4413.

- Himms-Hagen, J. (1999). Physiological roles of the leptin endocrine system: differences between mice and humans. *Critical Reviews in Clinical Laboratory Sciences* 36: 575-655.
- Hori, S., Hori, K., Ishigaki, T., Koyama, K., Kaya, M., Takeda, H., and Tsujita, J. (2001). Zucker obese rats are sensitive to weight-reducing effect and insensitive to oreogenic effect by cold exposure. *Journal of Thermal Biology* 26: 479-483.
- Houseknecht, K.L., Baile, C.A., Matteri, R.L., and Spurlock, M.E. (1998). The biology of leptin: a review. *Journal of Animal Science* 76: 1405-1420.
- Hsueh, W.C., Cole, S.A., Shuldiner, A.R., Beamer, B.A., Blangero, J., Hixson, J.E., MacCluer, J.W., and Mitchell, B.D. (2001). Interactions between variants in the beta3-adrenergic receptor and peroxisome proliferator-activated receptor-gamma2 genes and obesity. *Diabetes Care* 24: 672-677.
- Hube, F., Lietz, U., Igel, M., Jensen, P., Tornqvist, H., Joost, H., and Hauner, H. (1996). Difference In Leptin Mrna Levels Between Omental and Subcutaneous Abdominal Adipose Tissue From Obese Humans. *Hormone and Metabolic Research* 28: 690-693.
- ISO. (1992). Evaluation of thermal strain by physiological measurements: International Organisation for Standardisation.
- Jacobs, I., Martineau, L., and Vallerand, A.L. (1994). Thermoregulatory thermogenesis in humans during cold stress. *Exercise and Sport Sciences Reviews* 22: 221-250.
- Jansky, L., Vybiral, S., Stich, V., Sramek, P., Kvitek, J., Lesna, I., and Simeckova, M. (1997). Human humoral thermogenesis. *Annals of the New York Academy of Sciences* 813: 689-696.
- Janssen, J.A., Koper, J.W., Stolk, R.P., Englaro, P., Uitterlinden, A.G., Huang, Q., van Leeuwen, J.P., Blum, W.F., Attanasio, A.M., Pols, H.A., Grobbee, D.E., de Jong, F.H., and Lamberts, S.W. (1998). Lack of associations between serum leptin, a polymorphism in the gene for the beta 3-adrenergic receptor and glucose tolerance in the Dutch population. *Clinical Endocrinology* 49: 229-234.
- Jenkins, A.B., Samaras, K., Gordon, M.A., Snieder, H., Spector, T., and Campbell, L.V. (2001). Lack of heritability of circulating leptin concentration in humans after adjustment for body size and adiposity using a physiological approach. *International Journal of Obesity* 25: 1625-1632.
- Jequier, E. and Tappy, L. (1999). Regulation of body weight in humans. *Physiological Reviews* 79: 451-480.
- Jessen, K. (1980). An assessment of human regulatory nonshivering thermogenesis. *Acta Anaesthesiologica Scandinavica* 24: 138-143.
- Johnson, D.G., Hayward, J.S., Jacobs, T.P., Collis, M.L., Eckerson, J.D., and Williams, R.H. (1977). Plasma norepinephrine responses of man in cold water. *Journal of*

Applied Physiology: Respiratory, Environmental and Exercise Physiology 43: 216-220.

- Katch, F.I. and McArdle, W.D. (1973). Prediction of body density from simple anthropometric measurements in college-age men and women. *Human Biology* 45: 445-454.
- Katzmarzyk, P.T. and Leonard, W.R. (1998). Climatic influences on human body size and proportions: ecological adaptations and secular trends. *American Journal of Physical Anthropology* 106: 483-503.
- Kershaw, E.E. and Flier, J.S. (2004). Adipose tissue as an endocrine organ. *Journal of Clinical Endocrinology and Metabolism* 89: 2548-2556.
- Kolaczynski, J.W., Morales, L.M., Moore, J.J., Considine, R.V., Pietrzkowski, Z., Noto, P.F., Colberg, J., and Caro, J.F. (1994). A new technique for biopsy of human abdominal fat under local anaesthesia with Lidocaine. *International Journal of Obesity and Related Metabolic Disorders* 18: 161-166.
- Kolaczynski, J.W., Nyce, M.R., Considine, R.V., Boden, G., Nolan, J.J., Henry, R., Mudaliar, S.R., Olefsky, J., and Caro, J.F. (1996). Acute and chronic effects of insulin on leptin production in humans: Studies in vivo and in vitro. *Diabetes* 45: 699-701.
- Koska, J., Ksinantova, L., Sebkova, E., Kvetnansky, R., Klimes, I., Chrousos, G., and Pacak, K. (2002). Endocrine regulation of subcutaneous fat metabolism during cold exposure in humans. *Annals of the New York Academy of Sciences*. 967: 500-505.
- Kronfeld-Schor, N., Richardson, C., Silvia, B.A., Kunz, T.H., and Widmaier, E.P. (2000). Dissociation of leptin secretion and adiposity during prehibernatory fattening in little brown bats. *American Journal of Physiology - Regulatory Integrative & Comparative Physiology* 279: R1277-1281.
- Large, V., Reynisdottir, S., Eleborg, L., van Harmelen, V., Strommer, L., and Arner, P. (1997). Lipolysis in human fat cells obtained under local and general anesthesia. *International Journal of Obesity and Related Metabolic Disorders* 21: 78-82.
- Laughlin, G. and Yen, S. (1997). Hypoleptinemia In Women Athletes - Absence Of a Diurnal Rhythm With Amenorrhea. *Journal of Clinical Endocrinology and Metabolism* 82: 318-321.
- Leblanc (1957). Effect of environmental temperature on energy expenditure and caloric requirements. *Journal of Applied Physiology* 10: 281-283.
- Leblanc, J. (1954). Subcutaneous fat and skin temperature. *Can J Med Sci* 32: 354-358.
- Livingston, S.D., Nolan, R.W., Frim, J., Reed, L.D., and Limmer, R.E. (1987). A thermographic study of the effect of body composition and ambient temperature

- on the accuracy of mean skin temperature calculations. *European Journal of Applied Physiology and Occupational Physiology* 56: 120-125.
- Martineau, L. and Jacobs, I. (1989). Free fatty acid availability and temperature regulation in cold water. *Journal of Applied Physiology* 67: 2466-2472.
- Martinez, A.E., Perez, M., and Martinez, J.A. (1998). Induction of hypothermia, hypoglycemia and hyperinsulinemia after acute leptin immunoneutralization in overnight fasted mice. *International Journal of Molecular Medicine* 2: 681-683.
- Meyer, C., Robson, D., Rackovsky, N., Nadkarni, V., and Gerich, J. (1997). Role of the kidney in human leptin metabolism. *American Journal of Physiology* 273: E903-E907.
- Miell, J., Englaro, P., and Blum, W. (1996). Dexamethasone Induces an Acute and Sustained Rise In Circulating Leptin Levels In Normal Human Subjects. *Hormone and Metabolic Research* 28: 704-707.
- Moinat, M., Deng, C., Muzzin, P., Assimacopoulos-Jeannet, F., Seydoux, J., Dulloo, A.G., and Giacobino, J.P. (1995). Modulation of obese gene expression in rat brown and white adipose tissues. *FEBS Letters* 373: 131-134.
- Montague, C.T., Prins, J.B., Sanders, L., Digby, J.E., and O'Rahilly, S. (1997). Depot- and sex-specific differences in human leptin mRNA expression: implications for the control of regional fat distribution. *Diabetes* 46: 342-347.
- Morrison, S.D. (1981). Cold-specific feeding response of rats to cold exposure and energy density of body weight change. *Journal of Applied Physiology* 51: 327-334.
- Neale, M.C. (1997). Mx; Statistical Modeling. Box 710 MCV Richmond, VA, 23298, USA: Dept of Psychiatry. 4th Edition.
- Neale, M.C., Walters, E.E., Eaves, L.J., Maes, H.H., and Kendler, K.S. (1994). Multivariate genetic analysis of twin-family data on fears: Mx models. *Behavior Genetics* 24: 119-139.
- Nieminen, P., Asikainen, J., and Hyvarinen, H. (2001). Effects of seasonality and fasting on the plasma leptin and thyroxin levels of the raccoon dog (*Nyctereutes procyonoides*) and the blue fox (*Alopex lagopus*). *Journal of Experimental Zoology* 289: 109-118.
- Peino, R., Pineiro, V., Gualillo, O., Menendez, C., Brenlla, J., Casabiell, X., Dieguez, C., and Casanueva, F.F. (2000). Cold exposure inhibits leptin secretion in vitro by a direct and non-specific action on adipose tissue. *European Journal of Endocrinology* 142: 195-199.
- Pinkney, J.H., Coppack, S.W., and Mohamed-Ali, V. (1998). Effect of isoprenaline on plasma leptin and lipolysis in humans. *Clinical Endocrinology* 48: 407-411.

- Prisby, R., Glickman-Weiss, E.L., Nelson, A.G., and Caine, N. (1999). Thermal and metabolic responses of high and low fat women to cold water immersion. *Aviation Space and Environmental Medicine* 70: 887-891.
- Puerta, M., Abelenda, M., Rocha, M., and Trayhurn, P. (2002). Effect of acute cold exposure on the expression of the adiponectin, resistin and leptin genes in rat white and brown adipose tissues. *Hormone and Metabolic Research* 34: 629-634.
- Ramsay, T.G. (1996). Fat cells. *Endocrinology and Metabolism Clinics of North America* 25: 847-870.
- Rayner, D.V. and Trayhurn, P. (2001). Regulation of leptin production: sympathetic nervous system interactions. *Journal of Molecular Medicine* 79: 8-20.
- Regan, J.M. Human physiology responses to cold- water immersion: acute and repeated exposures (Doctor of Philosophy Dissertation): University of Wollongong, Australia, 1998.
- Ricci, M.R. and Fried, S.K. (1999). Isoproterenol decreases leptin expression in adipose tissue of obese humans. *Obesity Research* 7: 233-240.
- Ricci, M.R., Fried, S.K., and Mittleman, K.D. (2000). Acute cold exposure decreases plasma leptin in women. *Metabolism: Clinical and Experimental* 49: 421-423.
- Ricci, M.R., Lee, M.J., Russell, C.D., Wang, Y., Sullivan, S., Schneider, S.H., Brolin, R.E., and Fried, S.K. (2005). Isoproterenol decreases leptin release from rat and human adipose tissue through posttranscriptional mechanisms. *Am J Physiol Endocrinol Metab* 288: E798-804.
- Robert, D. (1953). Body weight, race and climate. *American Journal of Physical Anthropology* 11: 533-558.
- Roberts, D. *Climate and human variability*. Menlo Park, CA: Cummings Publishing Company, 1978.
- Rosenbaum, M., Nicolson, M., Hirsch, J., Heymsfield, S.B., Gallagher, D., Chu, F., and Leibel, R.L. (1996). Effects of gender, body composition, and menopause on plasma concentrations of leptin. *Journal of Clinical Endocrinology and Metabolism* 81: 3424-3427.
- Russell, C.D., Ricci, M.R., Brolin, R.E., Magill, E., and Fried, S.K. (2001). Regulation of the leptin content of obese human adipose tissue. *American Journal of Physiology - Endocrinology & Metabolism* 280: E399-E404.
- Savard, G.K., Cooper, K.E., Veale, W.L., and Malkinson, T.J. (1985). Peripheral blood flow during rewarming from mild hypothermia in humans. *Journal of Applied Physiology* 58: 4-13.

- Scriba, D., Aprath-Husmann, I., Blum, W.F., and Hauner, H. (2000). Catecholamines suppress leptin release from in vitro differentiated subcutaneous human adipocytes in primary culture via beta1- and beta2-adrenergic receptors. *European Journal of Endocrinology / European Federation of Endocrine Societies* 143: 439-445.
- Seidell, J.C., Bakker, C.J., and van der Kooy, K. (1990). Imaging techniques for measuring adipose-tissue distribution--a comparison between computed tomography and 1.5-T magnetic resonance. *American Journal of Clinical Nutrition* 51: 953-957.
- Simeckova, M., Jansky, L., Lesna, I., Vybiral, S., and Sramek, P.U.-. (2000). Role of beta adrenoceptors in metabolic and cardiovascular responses of cold exposed humans. *Journal of Thermal Biology* 25: 437-442.
- Simonsen, L., Bulow, J., and Madsen, J. (1994). Adipose tissue metabolism in humans determined by vein catheterization and microdialysis techniques [see comments]. *American Journal of Physiology*.
- Sinha, M.K., Ohannesian, J.P., Heiman, M.L., Kriauciunas, A., Stephens, T.W., Magosin, S., Marco, C., and Caro, J.F. (1996a). Nocturnal rise of leptin in lean, obese, and non-insulin-dependent diabetes mellitus subjects. *Journal of Clinical Investigation* 97: 1344-1347.
- Sinha, M.K., Sturis, J., Ohannesian, J., Magosin, S., Stephens, T., Heiman, M.L., Polonsky, K.S., and Caro, J.F. (1996b). Ultradian oscillations of leptin secretion in humans. *Biochemical and Biophysical Research Communications* 228: 733-738.
- Snitker, S., Macdonald, I., Ravussin, E., and Astrup, A. (2000). The sympathetic nervous system and obesity: role in aetiology and treatment. *Obesity Reviews*. 1: 5-15.
- Snitker, S., Nicolson, M., Shuldiner, A.R., Silver, K., and Ravussin, E. (1998). No effect of Trp64Arg beta3-adrenoceptor polymorphism on the plasma leptin concentration in Pima Indians. *Metabolism: Clinical and Experimental* 47: 1525-1527.
- Sramek, P., Simeckova, M., Jansky, L., Savlikova, J., and Vybiral, S. (2000). Human physiological responses to immersion into water of different temperatures. *European Journal of Applied Physiology* 81: 436-442.
- Sramek, P., Ulicny, B., Jansky, L., Hosek, V., Zeman, V., and Janakova, H. (1993). Changes of body fluids and ions in cold-adapted subjects. *Sports Med., Training and Rehab.* 4: 195-203.
- Stangl, K., Cascorbi, I., Laule, M., Stangl, V., Vogt, M., Ziemer, S., Roots, I., Wernecke, K., Baumann, G., and Hauner, H. (2000). Elevated serum leptin in patients with coronary artery disease: no association with the Trp64Arg

polymorphism of the beta3-adrenergic receptor. *International Journal of Obesity and Related Metabolic Disorders* 24: 369-375.

- Stehling, O., Doring, H., Nuesslein, H.B., Olbort, M., and Schmidt, I. (1997). Leptin does not reduce body fat content but augments cold defense abilities in thermoneutrally reared rat pups. *Pflugers Archiv. European Journal of Physiology* 434: 694-697.
- Strubbe, J.H. and van Dijk, G. (2002). The temporal organization of ingestive behaviour and its interaction with regulation of energy balance. *Neuroscience and Biobehavioral Reviews* 26: 485-498.
- Tikuisis, P., Gonzalez, R.R., and Pandolf, K.B. (1988). Thermoregulatory model for immersion of humans in cold water. *Journal of Applied Physiology* 64: 719-727.
- Trayhurn, P., Duncan, J.S., and Rayner, D.V. (1995). Acute cold-induced suppression of ob (obese) gene expression in white adipose tissue of mice: mediation by the sympathetic system. *Biochemical Journal* 311: 729-733.
- Trayhurn, P., Hoggard, N., Mercer, J.G., and Rayner, D.V. (1999). Leptin: fundamental aspects. *International Journal of Obesity and Related Metabolic Disorders* 23 Suppl 1: 22-28.
- Vallerand, A.L. and Jacobs, I. (1989). Rates of energy substrates utilization during human cold exposure. *European Journal of Applied Physiology and Occupational Physiology* 58: 873-878.
- van Baak, M.A., Hul, G.B., Toubro, S., Astrup, A., Gottesdiener, K.M., DeSmet, M., and Saris, W.H. (2002). Acute effect of L-796568, a novel beta 3-adrenergic receptor agonist, on energy expenditure in obese men. *Clinical Pharmacology & Therapeutics*. 71: 272-279.
- Van Harmelen, V., Reynisdottir, S., Eriksson, P., Thorne, A., Hoffstedt, J., Lonnqvist, F., and Arner, P. (1998). Leptin secretion from subcutaneous and visceral adipose tissue in women. *Diabetes* 47: 913-917.
- Vendrell, J., Gutierrez, C., Broch, M., Fernandez-Real, J.M., Aguilar, C., and Richart, C. (1998). Beta 3-adrenoreceptor gene polymorphism and leptin. Lack of relationship in type 2 diabetic patients. *Clinical Endocrinology* 49: 679-683.
- Wabitsch, M., Jensen, P., Blum, W., Christoffersen, C., Englaro, P., Heinze, E., Rascher, W., Teller, W., Tornqvist, H., and Hauner, H. (1996). Insulin and Cortisol Promote Leptin Production In Cultured Human Fat Cells. *Diabetes* 45: 1435-1438.
- Webb, P. (1986). Afterdrop of body temperature during rewarming: an alternative explanation. *Journal of Applied Physiology* 60: 385-390.

- Webb, P. (1992). Temperatures of skin, subcutaneous tissue, muscle and core in resting men in cold, comfortable and hot conditions. *European Journal of Applied Physiology and Occupational Physiology* 64: 471-476.
- Westerterp-Plantenga, M.S. (1999). Effects of extreme environments on food intake in human subjects. *Proceedings of the Nutrition Society* 58: 791-798.
- Weyer, C., Tataranni, P.A., Snitker, S., Danforth, E., Jr., and Ravussin, E. (1998). Increase in insulin action and fat oxidation after treatment with CL 316,243, a highly selective beta3-adrenoceptor agonist in humans. *Diabetes* 47: 1555-1561.
- White, L.J., Dressendorfer, R.H., Holland, E., McCoy, S.C., and Ferguson, M.A. (2005). Increased caloric intake soon after exercise in cold water. *Int J Sport Nutr Exerc Metab* 15: 38-47.
- Woods, S.C., Schwartz, M.W., Baskin, D.G., and Seeley, R.J. (2000). Food intake and the regulation of body weight. *Annual Review of Psychology* 51: 255-277.
- Yoshida, T., Hayashi, M., Monkawa, T., and Saruta, T. (1996). Regulation of obese mRNA expression by hormonal factors in primary cultures of rat adipocytes. *European Journal of Endocrinology / European Federation of Endocrine Societies* 135: 619-625.
- Zeyl, A., Regan, J.M., Patterson, M.J., Taylor, N.A.S., and Jenkins, A.B. (1998). The effects of repeated cold-water exposure on plasma leptin concentration in humans. *Diabetologia*, 41, p. A219.
- Zeyl, A., Stocks, J.M., Taylor, N.A.S., and Jenkins, A.B. (2001). Cold-induced decreases in human, circulating leptin, and in the subcutaneous adipose leptin secretion rate. *Proceedings of the Australian Physiological and Pharmacological Society.. Associated Conference: International Thermal Physiology Symposium. September 2nd- 6th, 2001. Wollongong, Australia.*, 32, p. 188P.
- Zhang, Kumar, S., Barnett, A.H., and Eggo, M.C. (1999). Intrinsic site-specific differences in the expression of leptin in human adipocytes and its autocrine effects on glucose uptake. *Journal of Clinical Endocrinology and Metabolism* 84: 2550-2556.
- Zhang, Proenca, R., Maffei, M., Barone, M., Leopold, L., and Friedman, J.M. (1994). Positional cloning of the mouse obese gene and its human homologue. *Nature* 372: 425-432.