

Chapter I. Overview

A. Introduction

Obesity is a complex, chronic disease requiring long-term weight management. Annual healthcare costs in the U.S. associated with obesity have reached nearly \$100 billion (Weiser et al., 1997). The past 30-40 years have seen an increased emphasis on the need to recognize obesity as a chronic condition, driven by evidence that obesity has a causal role in cardiovascular diseases, diabetes mellitus and osteoarthritis (Gordon et al., 1976; Colditz et al., 1995). The strong relationship between obesity and various disease states has led to a heavy demand for strategies that result in long-term weight loss. Obesity is due to an imbalance between caloric intake and caloric expenditure resulting in storage of excess calories as fat. Thus, interventions to treat obesity have focused on reducing caloric intake or increasing caloric expenditure. The standard nonpharmacologic interventions are dietary restriction and increased physical activity (exercise). Unfortunately, nonpharmacologic treatments such as dieting and exercise have low efficacy due to patient noncompliance (Wooley et al., 1991). Consequently, their replacement or supplementation by pharmacologic agents that reduce body weight have received much attention.

Drugs that have been investigated for the management of obesity act by two general mechanisms, they either decrease caloric intake and/or increase caloric expenditure. Drugs that reduce caloric intake are commonly called anorexic or hypophagic agents. The hypophagic agents comprise two pharmacologic classes: drugs that affect catecholaminergic system (the amphetamines, phentermine, mazindol, phenylpropralamine) and those that affect the serotonergic system (fenfluramine, fluoxetine, sertraline) (Weiser et al., 1997). One

of the most recently developed antiobesity agent, sibutramine affects both catecholaminergic and serotonergic neuronal systems (Connoley et al., 1995).

Drugs that increase caloric expenditure are called thermogenic drugs. Thermogenic agents increase metabolic rate. Drugs that increase metabolic rate include ephedrine (Pasquali et al., 1993) and experimental agents such as BRL 26830A, a beta-adrenoceptor agonist (Connacher et al., 1992). None of the thermogenic agents is currently approved by the FDA for weight control. Some of the appetite suppressive agents such as fenfluramine and sibutramine are also known to increase caloric expenditure (Connoley et al., 1999).

The most commonly prescribed appetite suppressant weight-loss medication in the 1950s and 1960s was amphetamine. However, the use of amphetamine had serious limitations due to the psychomotor stimulant properties and a high abuse potential (Bray, 1993). The other sympathomimetic agents (mazindol, phenylpropanolamine and phentermine) were approved by the FDA only for short-term treatment of obesity since tolerance to the anorexic agents develops during long-term therapy (Weintraub, 1992).

In an effort to overcome the limitations of amphetamine, yet retain the very potent antiobesity effect, fenfluramine, an amphetamine analog, was developed in the 1960s as an appetite suppressive agent. Fenfluramine was found to be devoid of the psychomotor stimulant and abuse potential of amphetamine (Rowland et al., 1986). The potency of fenfluramine coupled with the lack of psychostimulant side effects made fenfluramine one of the most widely prescribed antiobesity agents. In over 40 years of therapeutic use, more than 70 million patients worldwide have been treated by fenfluramine (Curzon et al., 1997).

Reports of serious cardiovascular side effects, pulmonary hypertension and cardiac valvular lesions (Fishman, 1999; Teramae et al., 2000) following fenfluramine treatment led

to the withdrawal of fenfluramine from the U.S. markets in 1997. But fenfluramine still serves as a prototype against which new agents are compared. Newer agents have been developed with the hope of producing a drug with the antiobesity effects of fenfluramine but without the toxic side effects.

In order to achieve the objective of developing antiobesity agents as potent and as efficacious as fenfluramine, it is important that the mechanisms by which fenfluramine produces weight loss be understood. However, the pharmacologic mechanisms by which fenfluramine reduces body weight are not completely elucidated. There are two general mechanisms by which a drug can produce weight loss, appetite suppression and thermogenic activity. Thus, research has focused on the possibility that fenfluramine may possess appetite suppressive and thermogenic effects.

Fenfluramine has been shown to be a potent appetite suppressive agent (Jackson et al., 1997; Curzon et al., 1997; Gibson et al., 1993; Oluyomi et al., 1994). However, appetite suppression alone does not account for the lowering of body weight since the appetite suppressive effect wanes after initial therapy, yet the weight loss persists. In rats, fenfluramine suppresses appetite during the first two weeks of treatment and then the effect diminishes and caloric intake returns to pretreatment levels. Despite normal caloric intake, weight loss persists (Arase et al., 1988). And, if fenfluramine treatment was terminated, the animals gained weight without increasing their food intake. This observation has led investigators to the conclusion that in addition to appetite suppression, fenfluramine must also affect energy expenditure.

In fact, it has been shown that fenfluramine increases metabolic rate in rats by increasing the activity of brown adipose tissue (BAT) (Rothwell et al., 1992; Rothwell et al.,

1987; Levitsky et al., 1986; Levitsky et al., 1992; Preston et al., 1990). BAT is a major organ of thermogenesis (Lupien et al., 1985; Lupien et al., 1986) in rodents. In human infants, BAT is important for body temperature maintenance (Himms-Hagan, 1990). However, in human adults, the function of BAT is not known. Nevertheless, there is evidence that part of the antiobesity effect of fenfluramine in humans might be related to increased metabolic rate. Fenfluramine appears to potentiate the expenditure of calories, whenever caloric expenditure increases (Levitsky et al., 1992).

Despite the fact that fenfluramine increases caloric expenditure, there is evidence in the literature that fenfluramine treatment results in a hypothermia (Cryan et al., 2000; Malberg et al., 1997). However, the mechanisms of fenfluramine-mediated hypothermia have not been characterized. Because body temperature regulation is intrinsically interrelated to caloric utilization (Carlisle et al., 1993; Klaus et al., 1998; Trayhurn et al., 1995), investigating the underlying mechanisms by which fenfluramine lowers body temperature could lead to an understanding of the mechanisms by which fenfluramine produces weight loss. Thus, the specific focus of the experiments in this dissertation was to investigate the mechanisms by which fenfluramine lowers body temperature.

The sympathetic nervous system plays a major role in the maintenance of body temperature. A major hypothesis of the thesis was that the hypothermia produced by fenfluramine could be due to dysregulation of the sympathetic nervous system mechanisms of thermoregulation. Thus, the hypothermia to fenfluramine could be due to impairment of sympathetic regulation of heat generation and/or heat conservation.

B. Literature Survey

The following survey of the literature is divided into three sections. In part one, the role of sympathetic nervous system in thermoregulation is described. In part two, the effects of fenfluramine on thermoregulation are discussed. In part three, the role of serotonin in thermoregulation is described since fenfluramine is a serotonin agonist and mimics serotonin in its pharmacologic actions.

1. The role of sympathetic nervous system in thermoregulation

The sympathetic nervous system plays a critical role in the maintenance of body core temperature (Landsberg et al., 1984; Maickel et al., 1967). Sympathetic neurons, via the release of norepinephrine (NE) and the consequent stimulation of alpha-adrenoceptors, promote heat conservation through cutaneous vascular constriction and piloerection (Kent et al., 1991; Kent et al., 1990). The sympathetic neurons also promote heat loss by cutaneous vasodilation (O'leary et al., 1985). NE stimulation of beta-adrenoceptors results in heat generation in BAT (Arch, 1989; Hsieh et al., 1957; Tsukazaki et al., 1995) and stimulation of the heart (Barney et al., 1980; Fregly et al., 1989; Sun et al., 1997) to allow for appropriate adjustments in cardiac output resulting from changes in metabolic rate and cutaneous blood flow. These actions of NE are augmented by the adrenal medullary catecholamines (Himms-Hagan, 1975), Fig. 1.

The role of the sympathetic nervous system in the maintenance of body temperature is clearly demonstrated when animals are exposed to a cold environment (Maickel et al., 1967). The sympathetic nervous system is activated when animals are exposed to a cold environment. When animals were maintained at an ambient temperature of 4⁰C, the plasma NE levels were increased indicating that the sympathetic

neurons were activated (Leduc, 1961; Vollmer, 1996; Picotti et al., 1980). In fact, direct sympathetic nerve recordings have indicated that sympathetic nerve impulse traffic to BAT was increased during cold exposure (Banet et al., 1978). Also, when animals are exposed to a cold environment NE turnover rates in the BAT, white adipose tissue and heart, increase (Young et al., 1982; Garafalo et al., 1996; Landsberg et al., 1984).

Blockade of the sympathetic nervous system and adrenal medullary mechanisms of thermoregulatory responses to cold exposure impairs the ability of an animal to maintain body temperature (Maickel et al., 1967; Ramey et al., 1957; Taylor, 1960; Wekstein, 1964). Chemical sympathectomy by treating animals with reserpine, blockade of the autonomic ganglia by ganglionic blocking agents chlorisondamine and pentolinium (Maickel et al., 1967; Picotti et al., 1980) and blockade of alpha-receptor mediated vasoconstrictor response by phentolamine results in hypothermia (Redfern et al., 1995). Mutant mice that lack NE and EPI are cold intolerant because they have impaired peripheral vasoconstriction and are unable to induce BAT thermogenesis (Thomas et al., 1999).

The next two sections discuss the sympathetic nervous system control of metabolic heat production by BAT and heat conservation by constriction of the cutaneous vasculature.

a. Sympathetic nervous system control of BAT thermogenesis

BAT is a major site of nonshivering thermogenesis in rodents (Himms-Hagan, 1995). The thermogenesis in BAT can be stimulated by both exposure to cold (nonshivering thermogenesis, NST) and by overeating (diet-induced thermogenesis, DIT) (Himms-Hagan, 1990), thus, BAT contributes to both thermal and energy balance respectively. Destruction of BAT causes obesity and cold intolerance (Klaus et al., 1998). Studies have demonstrated that the BAT thermogenesis is regulated by the sympathetic nervous system, via direct

noradrenergic innervation of the BAT cells (Smith et al., 1969). Sympathetic control of BAT thermogenesis is also evidenced by increased NE turnover rate in BAT during cold exposure and by induction of thermogenesis by NE infusions in BAT (McDonald et al., 1993). NE released from the sympathetic neurons act on beta-adrenoceptors, particularly of the beta-3 subtype and activates heat production (Lowell et al., 1997; Tanaka et al., 1995). Beta-3 agonists and nonspecific beta agonists increase heat production in BAT and beta-antagonists like propranolol block thermogenesis in BAT (Carlisle et al., 1992; Benzi et al., 1988).

Heat production in BAT involves a unique mitochondrial protein called the uncoupling protein (UCP), which forms a proton conductance pathway allowing cells to oxidize substrates without the oxidation process linked to ATP synthesis. The UCP in the mitochondrial membrane binds to GDP and other purine nucleotides. The level of GDP binding to BAT mitochondria has been a reliable measure of the activity of the proton conductance pathway and hence thermogenic activity of BAT. Cold exposure increases GDP binding to BAT by at least two-fold as revealed by studies conducted using the intrascapular BAT (IBAT), which is the most widely studied BAT depot (henceforth, IBAT will be mentioned as BAT since almost all the studies have examined this BAT depot) (Cageao et al., 1995; Trayhurn et al., 1987). Also, blood flow and glucose uptake by BAT increases during cold exposure due to increased metabolic activity of BAT (Foster et al., 1977; Ma et al., 1991). The thermogenic effect of BAT to cold exposure can be prevented by local denervation of the sympathetic nerves entering intrascapular BAT (Minokoshi et al., 1986b; Minokoshi et al., 1986a; Foster et al., 1981).

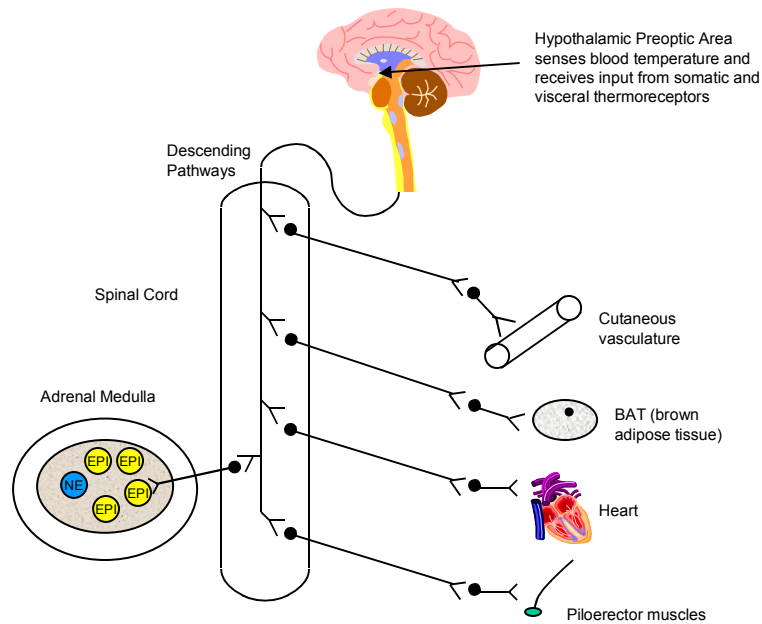


Fig. 1. Sympathetic pathways involved in thermoregulation. The sympathetic nervous system regulates heat conservation through cutaneous vasoconstriction and piloerection and heat generation through BAT activation. The sympathetic nervous system stimulates heart to adjust for changes in cardiac output resulting from changes in metabolic rate and cutaneous circulation. The sympathetic nervous system also controls adrenal release of catecholamines.

Brain centers that are involved in the control of BAT thermogenesis include the ventromedial hypothalamus, lateral hypothalamus, preoptic area and caudal raphe nucleus (Bamshad et al., 1999). Electrical stimulation of these areas activates BAT thermogenesis and this activation is blocked by local denervation of the sympathetic nerves to BAT (Amir, 1990; Minokoshi et al., 1986a).

b. Sympathetic nervous system control of cutaneous vascular responses

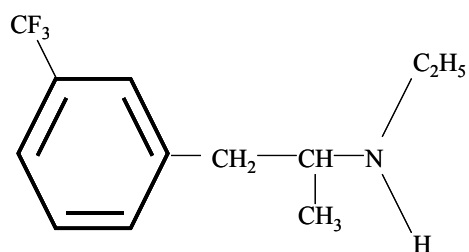
The cutaneous vasculature is an important thermoregulatory system. Cutaneous vasoconstriction prevents heat-loss and vasodilation promotes heat loss. The sympathetic nervous system regulates cutaneous vascular responses. The sympathetic neurons release NE, which interacts with the vascular smooth muscle alpha-adrenoreceptors to produce constriction. Withdrawal of sympathetic tone to the cutaneous vasculature leads to dilation (Redfern et al., 1995). Interference with sympathetic control of vascular tone leads to impairment of body temperature regulation. For example, treatment with alpha-receptor antagonists phentolamine and delequamine results in cutaneous vasodilation and hypothermia in rats (Kent et al., 1991; Redfern et al., 1995).

Rats are particularly suited for the study of cutaneous vascular responses because of the important role of the tail vasculature. The tail of the rat has frequently been used to study cutaneous vascular responses since it is a major thermoregulatory organ in the rat. About 20% of the heat loss in a rat occurs by sympathetically mediated increases in blood flow in the skin of the tail (O'leary et al., 1985; Redfern et al., 1995). The tail is well suited for dissipating heat since it lacks fur, is highly vascularized and has a relatively

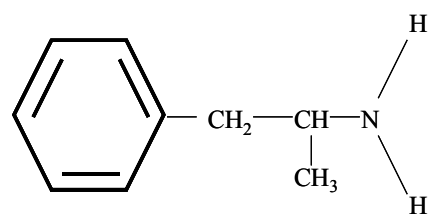
large surface area to volume ratio. Thus the dilation of the tail is important for heat loss when the environmental temperature is elevated and conversely constriction of this circulation is crucial for heat conservation when the ambient temperature is reduced. Many studies have measured tail surface temperature as an index of tail blood flow (Lin, 1978; Redfern et al., 1995). Blood flow to the tail and hence tail temperature is minimal at ambient temperatures below 25⁰C. At about 28-30⁰C, there is an abrupt increase in the blood flow suggestive of an on-off sequence of vasoconstriction and vasodilation within a very short period of time (Young et al., 1982).

The tail artery is thought to be controlled by a single class of sympathetic vasoconstrictive fibres. Pseudo-rabies virus tracing studies have revealed that the central cell groups that project to this sympathetic outflow lie in the ventral medulla, the preoptic area and the paraventricular nucleus of the hypothalamus. These cell groups project to the spinal cord where preganglionic fibres that innervate the tail vasculature originate (Smith et al., 1998b; Smith et al., 1998a). The postganglionic sympathetic fibres release NE, which activates alpha receptors, particularly alpha-2 receptors, mediating tonic sympathetic vasoconstriction (Redfern et al., 1995).

The foot of the rat is also an important site that regulates heat loss. However, it is difficult to obtain foot skin temperature of freely moving rats. The foot and tail have been shown to vasodilate simultaneously in response to various drugs (Lin, 1978; Lin et al., 1979). Like tail-skin temperature, foot temperature also rises abruptly at ambient temperatures above thermoneutrality (Gordon, 1990).



Fenfluramine



Amphetamine

Fig. 2. The chemical structures of fenfluramine and amphetamine. Fenfluramine is an amphetamine analog. However, fenfluramine lacks the psychostimulant and abuse potential of amphetamine. Fenfluramine affects serotonergic neurons whereas amphetamine affects noradrenergic and dopaminergic neurons as well.

2. Effects of fenfluramine on body temperature regulation

Fenfluramine is a derivative of amphetamine, Fig. 2. Fenfluramine acts as a serotonin agonist by stimulating serotonin release, blocking serotonin reuptake in to the nerve terminals and by directly acting on serotonergic receptors (Garattini et al., 1975; Raiteri et al., 1995; Berger et al., 1992), Fig. 3.

Antiobesity agents generally produce weight loss by decreasing appetite and/or increasing energy expenditure. There exists evidence that fenfluramine possesses both actions to produce weight loss. Fenfluramine acutely decreases caloric intake in both rats and humans (Jackson et al., 1997; Gibson et al., 1993; Oluyomi et al., 1994; Weiser et al., 1997). However, on prolonged treatment, the food intake returns to pre-drug levels, yet, the weight loss is maintained (Arase et al., 1989) (Lupien et al., 1985). These studies imply that fenfluramine also increases energy expenditure, i.e., it is thermogenic. Fenfluramine treatment could sustain long-term weight loss by increasing metabolic heat production.

Fenfluramine increases the metabolic rate acutely in rats as revealed by an increase in whole body oxygen consumption (Rothwell et al., 1992). In rodents, the thermogenic effect of fenfluramine is accounted for by its ability to increase the sympathetic neural drive to BAT (Rothwell et al., 1984; Preston et al., 1990). Despite the fact that fenfluramine is thermogenic, acute fenfluramine treatment has been shown to produce hypothermia (Cryan et al., 2000; Malberg et al., 1997). The expression of hypothermia following fenfluramine treatment depends on the ambient temperature at which the animals are maintained. At normal room temperature of 22⁰C, some studies

have reported that fenfluramine had no effect on body temperature (Sugrue, 1984) whereas other studies have shown that fenfluramine produces hypothermia at 22⁰C (Cryan et al., 2000). Though the effect of fenfluramine on body temperature in animals kept at 22⁰C remains controversial, at 4⁰C, a distinct hypothermia to fenfluramine treatment has been reported (Malberg et al., 1997; Preston et al., 1990).

The hypothermic effect of fenfluramine appears to be mediated by serotonin since the effect was attenuated by pretreatment with serotonin reuptake blocker sertraline, which blocks fenfluramine-mediated serotonin release (Cryan et al., 2000). The serotonin receptor antagonist metergoline also attenuates the hypothermia to fenfluramine (MacLeod et al., 1992; MacLeod et al., 1993). Serotonin-1A receptor antagonist cyclohexane carboxamide (WAY 100635) and serotonin-2C receptor antagonist benzofuran-2-carboxamide (RO 43-0440) were also able to attenuate the hypothermia to fenfluramine (Cryan et al., 2000).

However, the mechanism by which fenfluramine produces hypothermia remains to be elucidated. The hypothermia resulting from fenfluramine treatment could be due to decreased heat production and/or increased heat loss. The effects of fenfluramine on BAT thermogenesis, cutaneous vasodilation and total metabolic rate are reviewed in the following three sections.

a. Effects of fenfluramine on BAT thermogenesis

Fenfluramine increases metabolic rate acutely, in animal models as shown by increases in resting oxygen consumption, which are not associated with increases in physical activity (MacLeod et al., 1992; Preston et al., 1990; Rothwell et al., 1992). The increase in oxygen consumption produced by fenfluramine in rats is accounted for by its

ability to activate BAT. If fenfluramine-induced BAT activation is blocked, then fenfluramine does not increase oxygen consumption (Rothwell et al., 1987).

Fenfluramine stimulates BAT thermogenesis in rats. Fenfluramine increases the sympathetic neural drive to BAT as indicated by increased firing rates of sympathetic efferent neurons to BAT (Arase et al., 1988). Fenfluramine-induced activation of BAT is also indicated by increased blood flow to BAT (Ma et al., 1991; Foster et al., 1978) and increased GDP binding to BAT (Trayhurn et al., 1987) following fenfluramine administration. Central injections of fenfluramine in anesthetized rats also elicit an increase in intrascapular BAT temperature (Amir et al., 1991) indicating the thermogenic effect of fenfluramine on BAT. All these experiments were conducted at an ambient temperature of 22⁰C or at thermoneutrality (28⁰C). The effects of fenfluramine on BAT thermogenesis at cooler ambient temperatures are not known. It is not known whether the exacerbated hypothermia to fenfluramine treatment at cooler ambient temperatures is due to impairment of BAT thermogenesis at those temperatures.

Fenfluramine does not stimulate BAT thermogenesis directly, but via central sympathetic activation, since fenfluramine-mediated BAT activation was blocked when the sympathetic nerves to BAT were severed or when animals were pretreated with ganglionic blocker hexamethonium, which blocks sympathetic ganglionic transmission to BAT (Rothwell et al., 1984) . It is not known whether the activation of BAT by fenfluramine is mediated via serotonin.

b. Effects of fenfluramine on the cutaneous vasculature

Fenfluramine could produce hypothermia by increasing heat loss. Fenfluramine administration has been shown to produce heat loss in domestic fowls (MacLeod et al.,

1992). Although the effects of fenfluramine on heat loss pathways in rodents have not been studied, there are substantial reports that have shown that serotonin and certain serotonin agonists elicit hypothermia in rodents, associated with dilation of the cutaneous vasculature (Key et al., 1992)(Lin, 1978). Fluoxetine, a serotonin reuptake inhibitor, is known to increase tail and foot skin temperature of rats implying the involvement of serotonin in producing heat loss in rats (Lin, 1978). Also, serotonin 1A-receptor agonist 8-hydroxy-2-(di-n-propylamino)tetralin (8-OH DPAT) has been shown to induce cutaneous vasodilation (Oerther, 2000).

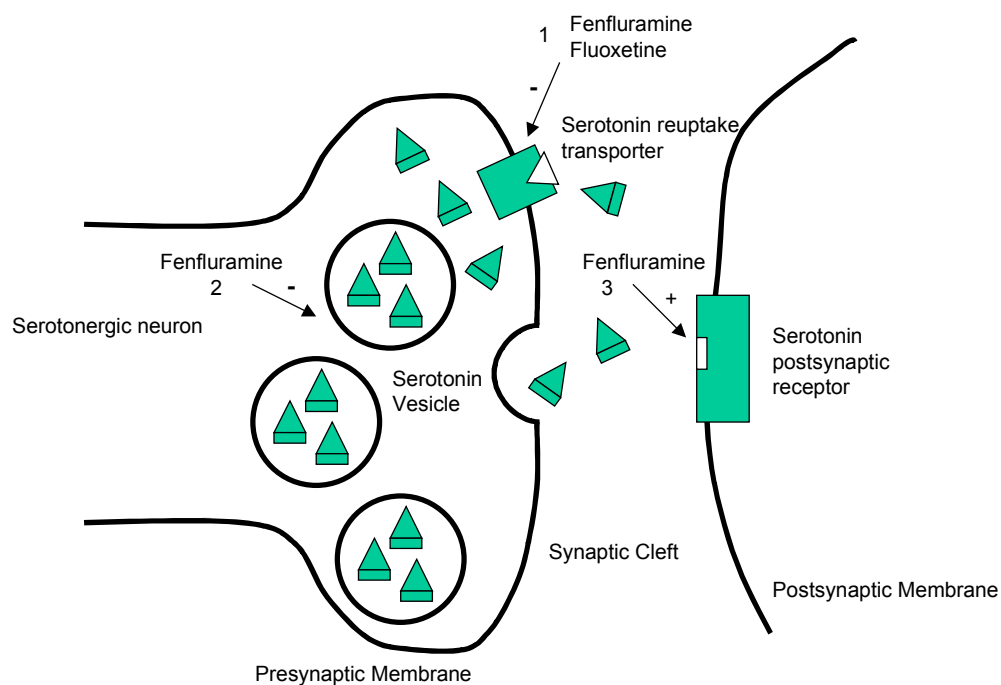


Fig. 3. The sites of action of fenfluramine. 1. Fenfluramine inhibits the reuptake of synaptic serotonin by blocking the reuptake transporter. 2. Fenfluramine disrupts serotonin storage in the vesicles and the unstored serotonin is released into the synapse through an outward carrier mediated transport. 3. Fenfluramine is also known to act on post-synaptic 5-HT receptors.

c. Effects of fenfluramine on metabolic rate

Fenfluramine is known to increase oxygen consumption, which is a measure of metabolic rate, in rats (Rothwell et al., 1992; Preston et al., 1990) kept at 22 and 28⁰C. The effect of fenfluramine on metabolic rate at cooler ambient temperatures has not been evaluated.

The increase in metabolic rate could be due to shivering thermogenesis or nonshivering thermogenesis through activation of BAT. The effect of fenfluramine on the metabolic rate in rats is clearly accounted for by its ability to increase BAT thermogenesis i.e., nonshivering thermogenesis, because blockade of fenfluramine-induced BAT activation by ganglionic blocker hexamethonium, also antagonizes the increase in oxygen consumption produced by fenfluramine (Rothwell et al., 1984). Furthermore, the thermogenic effect of fenfluramine clearly involves serotonergic systems since the increase in oxygen consumption following treatment with fenfluramine could be blocked by serotonin antagonists metergoline and methysergide (Rothwell et al., 1987).

3. The role of serotonin in thermoregulation

Numerous studies have revealed that serotonin is intimately involved in the regulation of body temperature (Sugimoto.Y et al., 1990) (Clark et al., 1986; Myers et al., 1978). Central injections of serotonin have been found to produce hypothermia (Brittain et al., 1967; Yamada et al., 1988) (Feldberg et al., 1967). Also, several agonists of serotonin, like selective serotonin reuptake inhibitor fluoxetine (Lin, 1978), which increases synaptic serotonin levels, serotonin-1A receptor agonist 8-OH DPAT (Lin et al., 1998), serotonin

releaser and reuptake inhibitor, fenfluramine and 3,4-methylene-dioxy-methamphetamine (MDMA) (Malberg et al., 1998) are known to produce hypothermia.

The hypothermic response to serotonin is thought to be mediated through activation of serotonin-1A receptors. The serotonin-induced hypothermia was attenuated by serotonin-1A receptor antagonist, pindolol (Yamada et al., 1988). And agonists at this receptor subtype, like, 8-OH-DPAT and ipsapirone, produce a dose-dependent hypothermia (Hjorth.S, 1985; Hillegaart, 1991). Also, as mentioned in the previous section, the hypothermia produced by fenfluramine was blocked by pretreating the animals with serotonin-1A receptor antagonist WAY-100635. The serotonin-2A/2C receptors have also been implicated in the hypothermic response to serotonin. The serotonin-2A/2C receptor agonist, 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI) also produces a dose dependent hypothermia (Salmi et al., 1998) and the antagonist RO 43-0440 attenuates the hypothermia to fenfluramine (Cryan et al., 2000).

The hypothermia resulting from treatment with serotonin and certain serotonin agonists is associated with the dilation of the cutaneous vasculature. Peripheral administration of fluoxetine produced an increase in temperature of the rat-tail skin and foot sole implying that fluoxetine produces cutaneous vasodilation (Lin, 1978). Serotonin-1A agonist-induced hypothermia was also associated with cutaneous vasodilation (Oerther, 2000). Also, the serotonin antagonist methysergide is known to block heat loss produced by fenfluramine administration, in the domestic fowl (MacLeod et al., 1992). These observations suggest that serotonin produces heat loss. In addition, the brain centers like the ventral medulla and raphe pallidus that control tail vascular responses contain serotonin immunoreactivity (Smith et al., 1998a).

The hypothermia to serotonin could also be due to a decrease in metabolic heat production. However, numerous studies have demonstrated that serotonin is thermogenic. Central serotonin injections in rats have increased the resting metabolic rates by 20% (Rothwell et al., 1987). These increases in metabolic rate are associated with elevated BAT activity and blood flow to BAT (Yoshimura et al., 1969). The effect of serotonin on BAT is mediated via the increased sympathetic stimulation of the tissue. Acute injections of serotonin in the paraventricular nucleus and the ventromedial hypothalamus of anesthetized rats were reported to increase the firing rate of sympathetic nerves to BAT (Sakaguchi et al., 1989). Also, the increase in oxygen consumption by serotonin was blocked by the ganglionic blocker hexamethonium (Rothwell et al., 1987). Anatomically, serotonergic neurons are associated with the central neural pathways, which includes the hypothalamus, raphe nuclei and medullary centers that project to BAT. Also, it has recently been shown that the premotor neurons to BAT, located in the raphe pallidus, are under the tonic inhibitory influence of the GABAergic neurons. Pharmacologic blockade of the GABA receptors has been shown to produce a significant increase in the postganglionic sympathetic firing in neurons innervating BAT. The increased neuronal firing was attenuated by the serotonin-1A receptor agonist 8-OH-DPAT, which inhibits the release of serotonin by acting on the presynaptic autoreceptors (Morrison et al., 1999). This observation concurs with the theory that increased central serotonin levels leads to activation of premotor neurons to BAT.

In summary, from the background information it is clear that fenfluramine produces hypothermia. However, the mechanisms by which fenfluramine produce hypothermia are not understood. The overall objective of this dissertation was to characterize the mechanism by which fenfluramine lowers body temperature. Therefore, experiments were designed first, to

assess the role of the sympathetic nervous system to body temperature maintenance and second, to determine if fenfluramine-induced hypothermia was due to dysregulation of the sympathetic nervous system control of BAT thermogenesis and/or cutaneous vasoconstriction. The specific objectives of the research are discussed in the following section.

C. Specific Objectives of the Research

The overall objective of this research was to characterize the mechanisms that mediate the hypothermic effect of fenfluramine. The effects of fenfluramine on body temperature, BAT thermogenesis, cutaneous vascular tone and total metabolic rate were studied in order to elucidate the hypothermic response to fenfluramine.

The specific objectives of the dissertation are

1. To Assess the Contribution of the Sympathetic Nervous System to the Maintenance of Body Temperature in Conscious Rats.

- a. To determine the effects of pharmacologic blockade of heat conservation and heat generation mechanisms on body temperature. This was achieved by treating animals with alpha-adrenoceptor blocker phentolamine, which blocks sympathetic nervous system-mediated cutaneous vasoconstriction, beta-adrenoceptor blocker propranolol, which blocks sympathetic nervous system-mediated BAT thermogenesis, alpha+beta-adrenoceptor blockers phentolamine + propranolol and the blockade of the autonomic ganglia by chlorisondamine.
- b. To determine if catecholamine contents of the adrenal glands and BAT could serve as an index of sympathetic activation. This was achieved by treating animals with

phentolamine, propranolol, phentolamine + propranolol, chlorisondamine and measuring adrenal and BAT catecholamine content.

2. To Evaluate the Effects of Fenfluramine on Sympathetic Nervous System

Regulation of Body Temperature.

- a. To determine the effects of fenfluramine on body temperature and BAT NE content. This was accomplished by treating animals with fenfluramine and measuring body temperature and BAT NE content.
- b. To determine if fenfluramine-induced BAT thermogenesis was mediated through activation of the sympathetic nervous system. This was accomplished by comparing the effects of fenfluramine on BAT NE content in animals treated with and without the ganglionic blocker, pentolinium.
- c. To determine if fenfluramine-induced BAT thermogenesis was mediated through release of serotonin. This was accomplished by comparing the effects of fenfluramine on BAT NE content in animals treated with and without serotonin reuptake inhibitor fluoxetine, which blocks fenfluramine-induced serotonin release.

3. To Evaluate the Effects of Fenfluramine on the Cutaneous Vasculature and Total Metabolic Heat Production.

- a. To determine the effects of fenfluramine on the cutaneous vasculature. This was achieved by treating animals with fenfluramine and measuring rat-tail skin temperature.
- b. To determine if the vasodilatory effect of fenfluramine was due to withdrawal of sympathetic vasoconstrictor tone. This was achieved by comparing the effects of fenfluramine on tail-skin temperature in animals treated with and without the

- ganglionic blocker, pentolinium, which removes the sympathetic vascular tone to the tail. The effect of fenfluramine on tail dilation was also studied at 28⁰C, a temperature at which the sympathetic constrictor tone to the tail is absent.
- c. To determine if the vasodilatory effect of fenfluramine was due to fenfluramine-induced serotonin release. This was achieved by comparing the effects of fenfluramine on tail-skin temperature in animals treated with and without fluoxetine, which blocks fenfluramine-mediated serotonin release.
 - d. To determine the effects of fenfluramine on total metabolic rate. This was achieved by treating animals with fenfluramine and measuring whole body consumption, which is an index of total metabolic rate.
 - e. To compare increases in metabolic rate due to BAT thermogenesis and non-BAT thermogenesis. This was achieved by treating animals with fenfluramine (BAT thermogenesis) and pentolinium (non-BAT thermogenesis) and measuring whole body oxygen consumption.

Chapter II. METHODS

All procedures for animal experimentation in this dissertation conform to the ethical standards set forth in the "Guide for the Care and Use of Laboratory Animals" and the "PHS Policy on Humane Care and Use of Laboratory Animals". The protocols were approved by the Animal Care and Use Committee of the University of Pittsburgh.

A. Care and Housing of Animals

Male Sprague-Dawley rats (Hilltop Laboratories, Scottsdale, PA) weighing 225-250g upon arrival were housed in pairs in a room maintained at $22 \pm 1^{\circ}\text{C}$ with a 12:12 hour light-dark cycle. Food and water were available *ad libitum*. A one-week acclimatization period was allowed before the rats were used for any surgical procedure or experiment. Animals in which surgery was performed, were housed individually and were allowed to recover for at least five days before being used for any experiment.

B. Measurement of Body Core Temperature by Temperature Telemetry and Computerized Data Acquisition System

Implantable temperature transmitters (Model #100-0001, VM-FH, Minimitters, Sunriver, OR) were used in these experiments. The transmitters weighed approximately 2 grams and had a sensitivity of 0.1°C . The transmitter transmits signal in the form of a series of short bursts of radio frequency energy to a receiver, which is connected to a computerized data acquisition system (Datacol, Minimitters, OR).

Prior to implantation, the transmitters were coated with warmed paraffin (Minimitters, Sunriver, OR) to prevent entry of fluid into the transmitter and to enhance biocompatibility.

Surgery was performed under aseptic conditions. Rats were anesthetized with pentobarbital sodium, 60 mg/kg, i.p., and temperature transmitters were implanted into the abdominal cavity via a midline incision. The incision was closed using surgical sutures. Following surgery, the animals were housed individually and a minimum of five days was allowed for recovery before experiments were performed.

When body temperature was measured immediately after surgery, the animals were hypothermic, an observation consistent with literature reports (Shimizu et al., 1991). However, 24 hours post-surgery, body temperature returned to normal levels. Therefore it was considered that the animals had recovered from the surgery and could maintain body temperature. In order to test this we conducted a preliminary study. The animals were treated with a ganglionic blocker, chlorisondamine, 3 mg/kg, i.p., which has been shown by others not to have affected body temperature maintenance in spite of blocking the sympathetic nervous system inputs to body temperature homeostasis, at normal ambient temperatures, 22°C (Maickel et al., 1967).

The animals were studied a day after the transmitter implantation surgery. They were treated with ganglionic blocker, chlorisondamine. The animals became hypothermic and the body temperature remained lower than control values throughout the four-hour observation period (Fig. 4). However, subsequently, when animals were allowed to recover for five days and then treated with chlorisondamine, the animals were able to maintain their body core

temperature (Fig. 5). Therefore, in all subsequent experiments, the animals were studied after a minimum of five days after the surgery.

On the day of the experiment, the animals were transferred to plastic observation boxes, which were placed on temperature receivers. Throughout the experimental period, the temperature was recorded at one-minute intervals via a computerized data acquisition system.

Experiments involving cold exposure were conducted in a walk-in cold room whose temperature could be controlled. The cold room is within the same laboratory and immediately adjacent to the area where other animals were kept. Entry to the cold room is via a glass door, which permits the lighting conditions to be equivalent for all animals (Fig. 7).

C. Measurement of Tail Skin Temperature Using Thermocouples in a Controlled Temperature Environment

Male Sprague-Dawley rats weighing 225-250 g upon arrival were housed in pairs for a week. Temperature transmitters were surgically implanted in the abdominal cavity and the animals were allowed a minimum of five days to recover. On the day of the experiment, the animals were transferred to plastic observation boxes and were attached to a tethering system (Harvard Apparatus, MA) that allowed free movement. A thermocouple (Omega, CT) was attached to the dorsal skin of the tail, approximately 7 cm from the tip, using surgical tape as described by Redfern et al (Fig. 6)(Redfern et al., 1995). The electrical leads were then passed to the back of the neck and through the wire spring of the tethering device. The animals were transferred to the

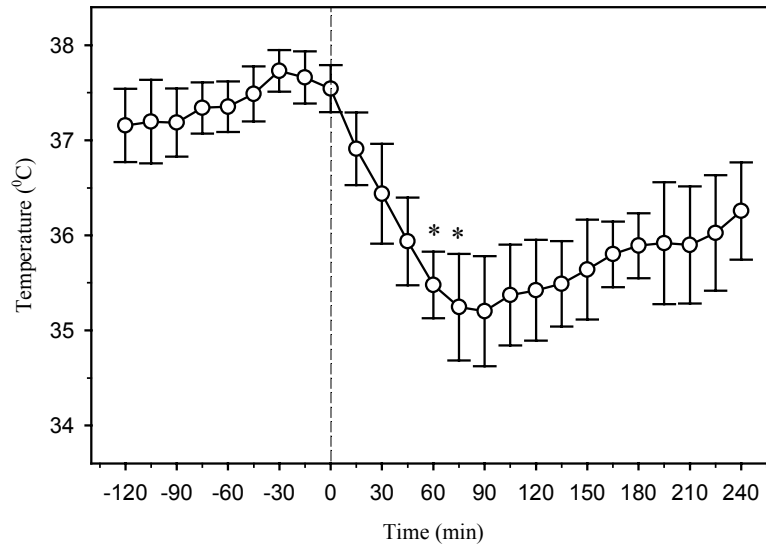


Fig. 4. Effect of chlorisondamine, 3mg/kg, i.p., on body temperature of rats 24-hours after surgery to implant temperature transmitter. The rats were allowed to recover for 24 hours after surgery. Chlorisondamine was administered after a two-hour baseline period, at time “0”. Asterisks indicate a significant difference from the baseline temperature, $p < 0.01$ (Repeated measures ANOVA, Bonferoni t-test).

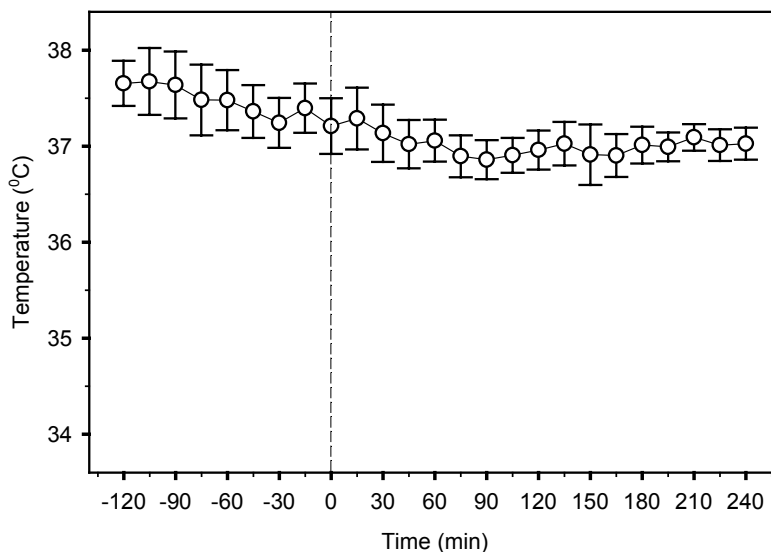


Fig. 5. Effect of chlorisondamine, 3 mg/kg, i.p., on body temperature of rats after five days of recovery following surgery to implant temperature transmitter. The rats were allowed to recover for five days following the surgery. Chlorisondamine was administered after a two-hour baseline period, at time “0”.

walk-in cold room maintained at an ambient temperature of 16-18⁰C and they were then placed on the temperature receiver to obtain a continuous record of the body core temperature. The tail temperature was recorded every minute in a hand held thermocouple thermometer (Omega, CT).

D. Measurement of Oxygen Consumption Using Open Circuit Calorimetry

Oxygen consumption (VO_2) was determined in an open circuit calorimeter (Oxymax, Columbus, OH). The calorimeter was placed in the walk-in cold room maintained at an ambient temperature of 16-17⁰C. Male rats with in-dwelling temperature transmitters were placed in the plastic metabolic test chamber through which a known flow of air, was passed. The system was calibrated with a known concentration of oxygen and carbon dioxide before each experiment. The system monitors oxygen and carbon dioxide gas fractions at both the inlet and output port of the test chamber. The gas fraction and flow measurements are used to compute oxygen consumption and carbon dioxide production. The oxygen consumption was measured every minute using a computerized data acquisition system.

E. Collection of Tissue Samples for Biochemical Analysis

Tissues (adrenal glands, intrascapular brown adipose tissue, white adipose tissue, atria and ventricles) were excised and frozen (-70⁰C) for later analysis of catecholamines. Tissues were homogenized in 0.1 N perchloric acid containing EDTA and sodium metabisulfite. The volume of homogenization for each tissue was as follows: adrenal glands, 2 ml/gland, atria, 1.5 ml/tissue, ventricles, intrascapular brown adipose tissue and

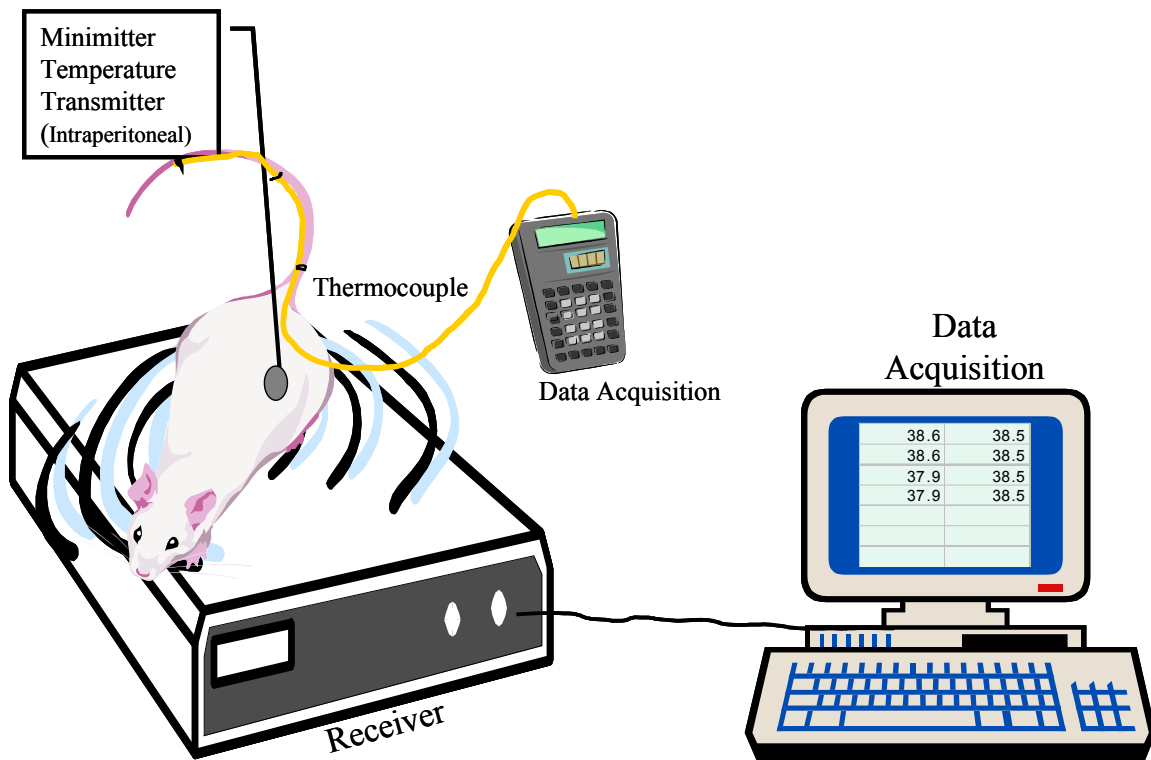


Fig. 6. Measurement of body temperature using temperature telemetry and a computerized data acquisition system and tail skin temperature using thermocouples. Body temperature was measured using temperature transmitters (Minimitters, OR) that were surgically implanted in the abdominal cavity of the rats. The data was collected using a computerized system. The tail skin temperature was measured by attaching thermocouples to the surface of the tail. The data was collected using a hand held thermometer.

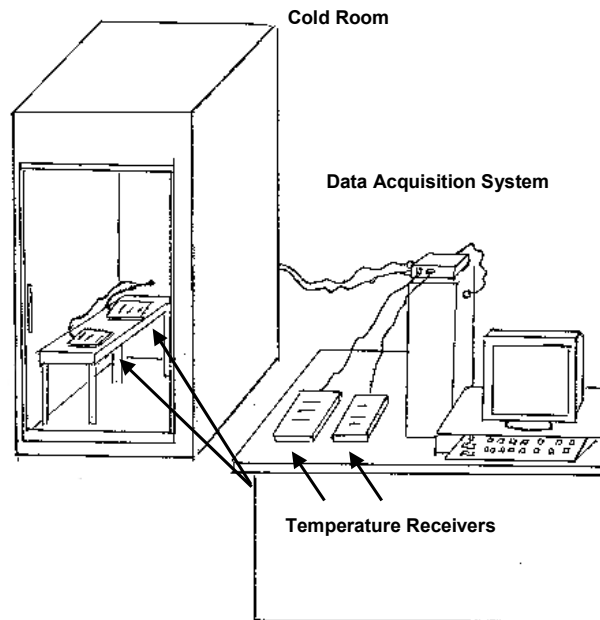


Fig. 7. Laboratory arrangement for performing experiments in a temperature-controlled environment. The temperature of the walk-in cold room could be varied from 28 to 0°C. The entrance to the cold room was via a glass door that permitted lighting conditions to be equivalent for animals inside and outside the cold room.

white adipose tissue, 200 mg tissue/ml. Homogenization was performed on ice for 30 seconds with a polytron homogenizer (Brinkmann Inst., Westbury, NY). Homogenates were centrifuged ($12,000 \times g$ for 15 minutes at 4°C) and the resultant supernatants collected and stored at -70°C for further biochemical analysis.

F. Measurement of Catecholamines by High Performance Liquid Chromatography (HPLC)

Catecholamines were assayed using high performance liquid chromatography (HPLC) with electrochemical detection (Waters, Marlborough, MA) by modification of existing methods (Weicker et al., 1984).

Extraction of catecholamines from tissue homogenates involved adsorption onto aluminium oxide (Woelm Neutral activity Grade 6). Sample aliquots (100-200 μl , depending on expected concentrations) were placed in 1.5 ml capped propylene tubes along with 10 mg of alumina. The internal standard, 3,4-dihydroxybenzylamine, was added to each sample, and the pH was adjusted to 8.6 with 1M tris(hydroxymethyl)aminomethane (Tris). The tubes were capped and shaken vigorously in a mechanical shaker for 10 minutes and centrifuged (12,000 RPM) for 2 minutes. The resulting pellet was washed 3 times with 600 μl of Tris (pH 7); catecholamines were desorbed with 100 μl of 0.1N perchloric acid through gentle mixing in a microtube shaker (TOMY, Peninsula Labs., CA) for 30 seconds. An appropriate volume of the eluate was immediately injected on to the HPLC system. Supernatants from adrenal glands did not require extraction, and were directly injected on to the HPLC system following dilution with perchloric acid (1:100).

Extracted catecholamines were separated on a 5 μ m, 3.9 x 150 mm C18 reverse-phase column (Waters, Marlborough, MA). The mobile phase consisted of sodium acetate (50 mM), citric acid monohydrate (20 mM), sodium-1-octane-sulfonate (2 mM), di-n-butylamine (1.0 mM), disodium EDTA (0.1 mM), and methanol (4%). Catecholamines were oxidized during exposure to a glassy carbon electrode set at a potential of 0.6 V versus Ag/AgCl. Data were acquired using ChromPerfect software (Justice Innovations, Mountain View, CA) and calculations were based on peak areas. The limit of quantitation for NE and EPI was 50 pg/ml. The recovery of catecholamines from alumina extraction was 65-70 %.

G. Statistics

Results are presented as mean \pm S.E. The presence of statistically significant differences was determined using Sigma Stat Statistical Software (Jandel Scientific, San Rafael, CA). Normality test was first performed to verify the nature of the distribution. The difference between multiple groups of means was assessed using analysis of variance (ANOVA). If ANOVA revealed a statistically significant difference, post hoc pairwise comparisons of means were done using the Bonferoni t-test. A repeated measures ANOVA was conducted for contrasts of groups in which multiple measurements were made over time. Post hoc contrasts were done using the Bonferoni t-test. A statistically significant effect was accepted when $p < 0.05$.

The size of the experimental groups was 7 rats/group. The group size is based on the variance estimates and power function calculations using preliminary data. The power of the test was calculated by the statistical software whenever data was analyzed.

Chapter III. Pharmacologic Assessment of the Contribution of the Sympathetic Nervous System to the Maintenance of Body Temperature in Conscious Rats

The overall goal of this thesis was to characterize the hypothermia to fenfluramine treatment. We hypothesized that fenfluramine might influence the sympathetic nervous system in such a way that the temperature homeostasis would be modified. Thus, the hypothermia to fenfluramine could be due to interference with sympathetically mediated heat generation or heat conservation. Despite the general acceptance that the sympathetic nervous system mediated responses are important to thermoregulation, there is not much evidence in the literature that equivocally demonstrates that disruption of sympathetic mechanisms of heat generation or conservation would lead to deficits in body temperature. Therefore, experiments in this chapter were designed to quantitatively assess the contribution of the sympathetic nervous system to the maintenance of body temperature.

The sympathetic nervous system is involved in the control of homeostatic adjustments that are initiated to maintain body temperature as discussed in detail in chapter I. In order to evaluate the contribution of the sympathetic nervous system mechanisms of heat generation and heat conservation to maintain body temperature, it was determined if pharmacologic blockade of the thermoregulatory actions of the sympathetic nervous system would result in a measurable decrease in body temperature. Therefore, the effects of the following pharmacological agents on body temperature maintenance were determined: phentolamine, an alpha-adrenoceptor blocking agent, was used to prevent sympathetic nervous system mediated cutaneous vasoconstriction. Propranolol, a beta-adrenoceptor blocking agent, was used to prevent sympathetically mediated thermogenesis, phentolamine plus propranolol to block both sympathetically mediated heat conservation and generation

respectively and chlorisondamine, a ganglionic blocker, was used to block sympathetic control of heat generation and conservation.

Body core temperature was recorded continuously, since body temperature is a direct measure of thermoregulation. The animals were conscious and temperature artifacts due to handling were prevented by using temperature telemetry as discussed in detail in chapter II. Measurement of body temperature using indwelling temperature transmitters is an improvement over prior studies because previous studies have measured body temperature using rectal thermometers, the use of which could lead to stress related increases in body temperature. Another advantage of using the temperature transmitters was that it also provided a measure of locomotor activity. Locomotor activity was measured in our experiments in order to determine if the animals increased physical activity to compensate for blockade of other thermoregulatory mechanisms.

Tissue catecholamine contents of the adrenal glands and BAT were measured in our experiments as an index of sympathetic activity. A previous study in our laboratory had indicated that adrenal catecholamine content was depleted during cold exposure (Vollmer et al., 1992) and a report from another laboratory had indicated that BAT NE content was also reduced during cold exposure (Brito et al., 1998). These reports suggested that the tissue NE content was attenuated due to sympathetic activation during cold exposure.

The measurements were conducted at two different ambient temperatures, 22 and 4°C. The experiment was conducted at normal room temperature of 22°C. And 4°C, a standard cold ambient temperature was used as a challenge to thermoregulatory mechanisms. The 4°C ambient temperature is known to activate the sympathetic nervous system and so the effect of the sympathetic nervous system on its effector organs would be more pronounced

(Daly et al., 1992; Young et al., 1988). Therefore drugs that affect the sympathetic mechanisms of body temperature maintenance would be expected to have a greater impact at 4⁰C.

A. Protocols

Male Sprague-Dawley rats with implanted temperature transmitters were used in this study. Half of the rats were studied at a laboratory room temperature of 22⁰C and the other half was exposed to a cold environment of 4⁰C. Five treatment groups (n=7/group) were investigated at 22 and 4⁰C. The treatments were: saline, 1ml/kg, i.p., phentolamine, 2 mg/kg, i.p., propranolol, 3 mg/kg, i.p., phentolamine, 2 mg/kg, i.p. plus propranolol, 3 mg/kg i.p. and chlorisondamine, 3 mg/kg, i.p. Doses of propranolol and chlorisondamine were selected based on previous studies conducted in our laboratory and by other investigators, in which it was demonstrated that sympathetic neuronal responses were more than 90% abolished for the time frame in which the current studies were conducted (Bush et al., 1983; Deitchman et al., 1980). The dose of phentolamine, 2mg/kg, i.p., was shown, by other investigators, to cause cutaneous vasodilation in rats (Lin et al., 1979).

In all groups, baseline body temperature was recorded for two hours prior to treatment. After treatment, the animals were monitored for four hours. During the six-hour experimental period, temperature and locomotor activity data was recorded at one-minute intervals and averaged every 15 minutes via a computerized data acquisition system. At the completion of the experiment, the animals were anesthetized with pentobarbital, 60 mg/kg, i.p. and intrascapular BAT and adrenal glands were removed and frozen (-70⁰C) for later analysis of catecholamines. Catecholamines were analyzed using HPLC.

Statistical Analyses

Results are presented as mean \pm S.E. The differences in multiple groups of animals maintained at 22 and 4⁰C for individual parameters such as BAT and adrenal catecholamine contents and activity data, were assessed using two-way analysis of variance (ANOVA). The differences in multiple measurements taken over a period of time were assessed using repeated measures ANOVA. If ANOVA revealed a statistically significant difference, post hoc pairwise comparisons of groups were done using Bonferoni t-test. A statistically significant effect was accepted when $p < 0.05$.

B. Results

1. Effects of saline, 1ml/kg, i.p., on body temperature, locomotor activity, adrenal and BAT catecholamine content.

During the first two hours of the experimental protocol (baseline period), there was no difference in the body temperature between the two groups of animals maintained at 22⁰C and 4⁰C, Fig. 8, left panel. Saline treatment did not affect body temperature maintenance in animals kept at 22 and 4⁰C, Fig. 8, left panel. Also, locomotor activity was not affected by saline treatment, Fig. 8, right panel.

Saline treatment did not affect adrenal norepinephrine (NE) or epinephrine (EPI) content of rats kept at 4⁰C when compared to rats kept at 22⁰C, Fig. 9, left panel. Also, saline treatment did not affect BAT norepinephrine (NE) content of rats kept at 4⁰C when compared to rats kept at 22⁰C, Fig. 9, right panel.

2. Effects of phentolamine, 2 mg/kg, i.p., on body temperature, locomotor activity, adrenal and BAT catecholamine content.

During the first two hours of the experimental protocol (baseline period), there was no difference in the body temperature between the two groups of animals maintained at 22⁰C and 4⁰C, Fig. 10, left panel. The body temperature of animals treated with phentolamine at 22 and 4⁰C were not different, Fig. 10, left panel. Phentolamine treatment at 22⁰C increased locomotor activity when compared to saline treated controls, $p < 0.001$ (Student t-test). However, when the activity of all the groups of animals were analyzed using ANOVA, there was no statistical difference, Fig. 10, right panel.

Phentolamine treatment did not affect adrenal NE or EPI content, Fig. 11, left panel. However, BAT NE content was significantly decreased (-57 %) in the cold exposed phentolamine treated animals, $p < 0.001$ (Two-way ANOVA). BAT NE content of the group of animals treated with phentolamine and kept at 22⁰C was significantly different from the group of animals treated with phentolamine and kept at 4⁰C (Bonferoni t-test), Fig. 11, right panel.

3. Effects of propranolol, 3 mg/kg, i.p., on body temperature, locomotor activity, adrenal and BAT catecholamine content.

During the first two hours of the experimental protocol (baseline period), there was no difference in the body temperature between the two groups of animals maintained at 22⁰C and 4⁰C, Fig. 12, left panel. Propranolol treatment did not affect body temperature maintenance in animals kept at 22⁰C. However, treatment of animals with

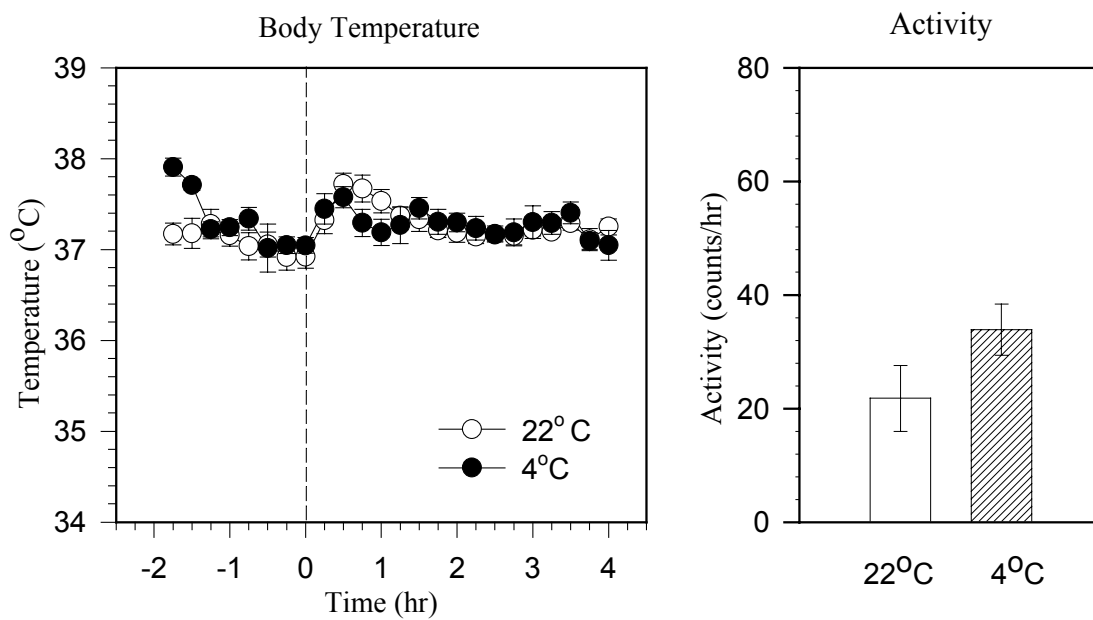


Fig. 8. Effects of saline, 1ml/kg, i.p., on body temperature and locomotor activity of rats kept at 22°C (n=7) and 4°C (n=7). Saline was administered at time “0”. Saline treatment did not affect body temperature maintenance and locomotor activity.

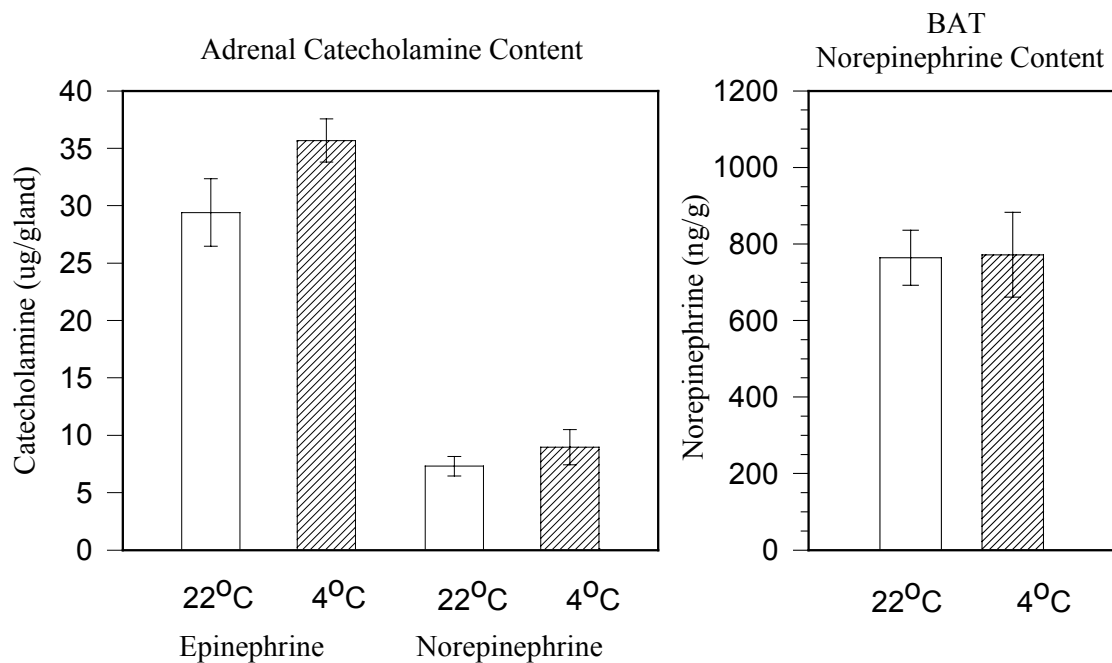


Fig. 9. Effects of saline, 1ml/kg, i.p., on adrenal epinephrine and norepinephrine (NE) content and brown adipose tissue (BAT) NE content in rats kept at 22°C (n=7) and 4°C (n=7). The animals were treated with saline and sacrificed 4 hours later. The adrenal and BAT catecholamine content was not different between the two groups.

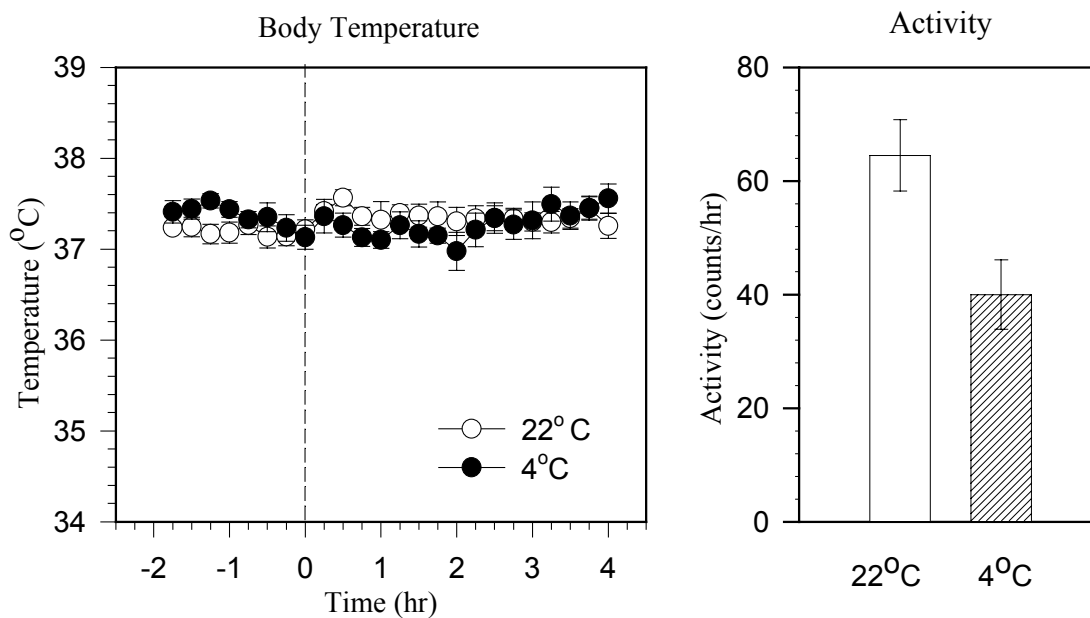


Fig. 10. Effects of phentolamine, 2mg/kg, i.p., on body temperature and locomotor activity of rats kept at 22°C (n=7) and 4°C (n=7). Phentolamine was administered at time "0". Body temperature remained constant throughout the observation period. The locomotor activity between the two groups was not significantly different.

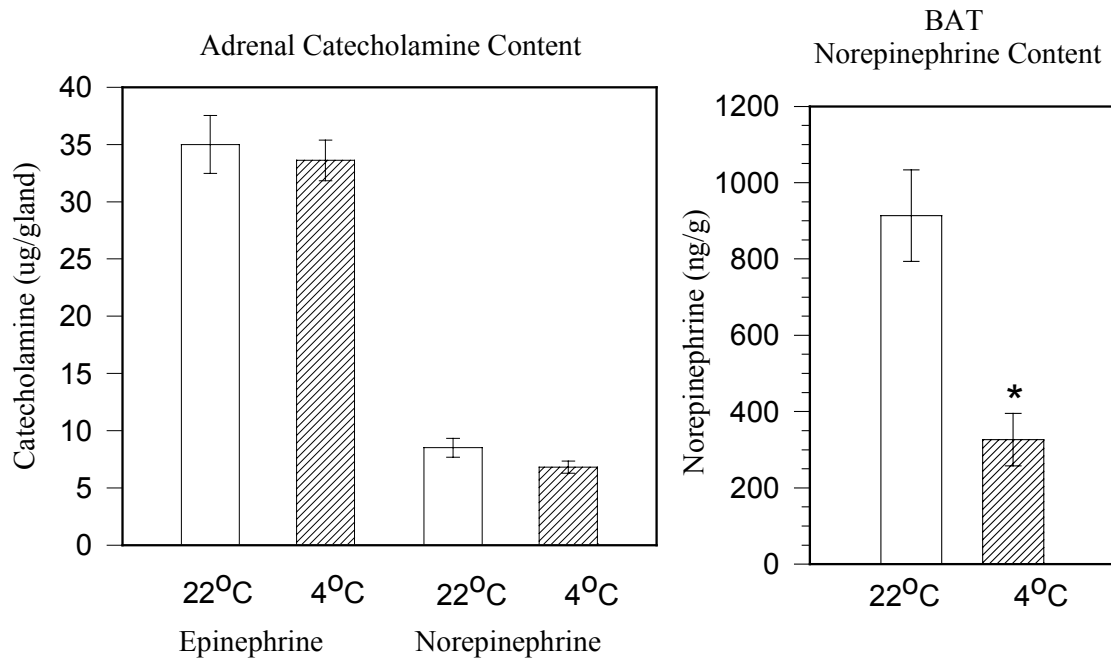


Fig. 11. Effects of phentolamine, 2mg/kg, i.p., on adrenal epinephrine and norepinephrine (NE) content and brown adipose tissue (BAT) NE content in rats kept at 22°C (n=7) and 4°C (n=7). The animals were treated with phentolamine and sacrificed 4 hours later. The adrenal catecholamine content between the two groups was not different. However, BAT of the cold exposed animals showed a significant depletion of its NE content. Two way ANOVA indicated that the groups are significantly different, $p < 0.001$. An asterisks indicate that there is a significant difference in BAT NE content between the phentolamine treated group kept at 22°C and the phentolamine treated group kept at 4°C, Bonferoni t-test.

propranolol caused body temperature to rise in the group kept at 4⁰C, $p < 0.01$ (Repeated measures ANOVA). The body temperature was significantly elevated after 2.5 hours post-treatment. The body temperature of the group of animals treated with propranolol and kept at 22⁰C was significantly different from the group of animals treated with propranolol and kept at 4⁰C (Bonferoni t-test), Fig. 12, left panel. Propranolol treatment did not affect locomotor activity, Fig. 12, right panel.

Propranolol treatment did not affect adrenal NE or EPI content, Fig. 13, left panel and BAT NE content, Fig. 13, right panel.

4. Effects of phentolamine, 2 mg/kg, i.p., and propranolol, 3 mg/kg, i.p., on body temperature, locomotor activity, adrenal and BAT catecholamine content.

During the first two hours of the experimental protocol (baseline period), there was no difference in the body temperature between the two groups of animals maintained at 22⁰C and 4⁰C, Fig. 14, left panel. Phentolamine + propranolol treatment did not affect body temperature maintenance in animals kept at 22⁰C. Cold exposed animals that received combined phentolamine and propranolol treatment showed a significant decline in body temperature that was maximal approximately 45 minutes after administration, $p < 0.01$ (Repeated measures ANOVA). Body temperature recovered to pretreatment levels at the end of the four-hour observation period. The body temperature of the group of animals treated with phentolamine + propranolol and kept at 22⁰C was significantly different from the group of animals treated with phentolamine + propranolol and kept at 4⁰C (Bonferoni t-test), Fig. 14, left panel. Phentolamine + propranolol treatment did not affect locomotor activity, Fig. 14, right panel.

Phentolamine + propranolol treatment did not affect adrenal NE or EPI content of rats kept at 4⁰C when compared to rats kept at 22⁰C, Fig. 15, left panel. Cold exposed animals that received the combined treatment also showed a very substantial reduction in BAT NE content (-97%), $p < 0.001$ (Two-way ANOVA). The BAT NE content of the group of animals treated with phentolamine + propranolol and kept at 22⁰C was significantly different from the group of animals treated with phentolamine + propranolol and kept at 4⁰C (Bonferoni t-test), Fig. 15, right panel.

5. Effects of chlorisondamine, 3 mg/kg, i.p., on body temperature, locomotor activity, adrenal and BAT catecholamine content.

During the first two hours of the experimental protocol (baseline period), there was no difference in the body temperature between the two groups of animals maintained at 22⁰C and 4⁰C, Fig. 16, left panel. Chlorisondamine treatment did not affect body temperature maintenance in animals kept at 22⁰C. The group of cold exposed animals treated with chlorisondamine showed a significant decline in body temperature, $p < 0.01$ (Repeated measures ANOVA). The peak decline in core temperature occurred one hour after administration and temperature recovered to pretreatment levels by the end of the experimental period. The body temperature of the group of animals treated with chlorisondamine and kept at 22⁰C was significantly different from the group of animals treated with chlorisondamine and kept at 4⁰C (Bonferoni t-test), Fig. 16, left panel. Chlorisondamine treatment did not affect locomotor activity, Fig. 16, right panel.

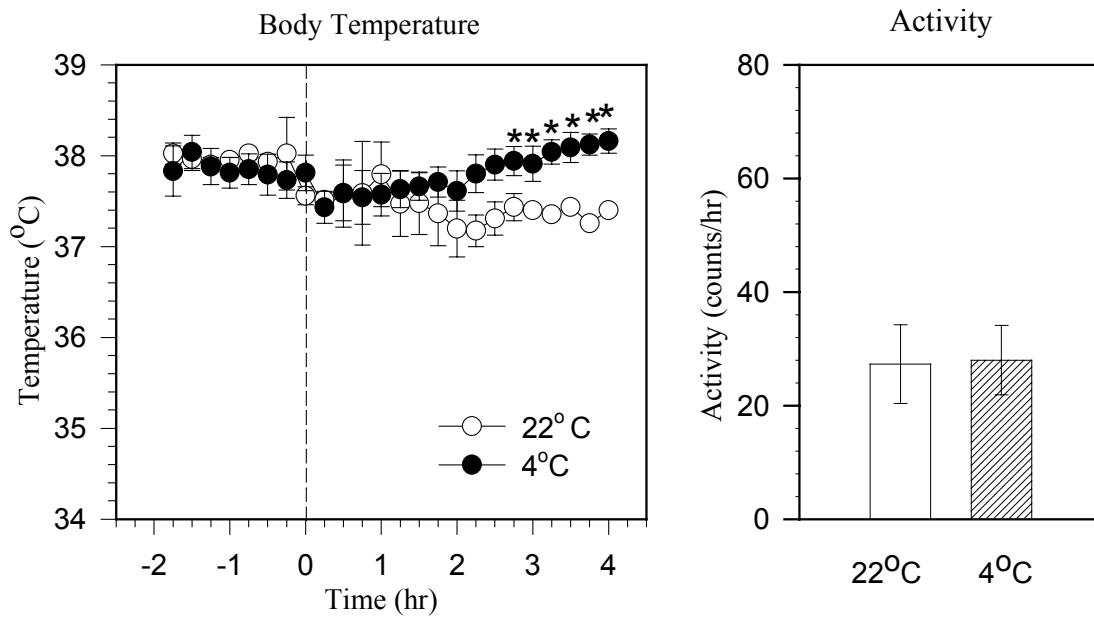


Fig. 12. Effects of propranolol, 3 mg/kg, i.p., on body temperature and locomotor activity of rats kept at 22°C (n=7) and 4°C (n=7). Propranolol was administered at time "0". Repeated measures ANOVA indicated that the body temperature of the groups was significantly different, $p < 0.01$. Asterisks indicate that there is a significant difference between the two groups at specific time points, Bonferoni t-test. The locomotor activity was not different between the two groups.

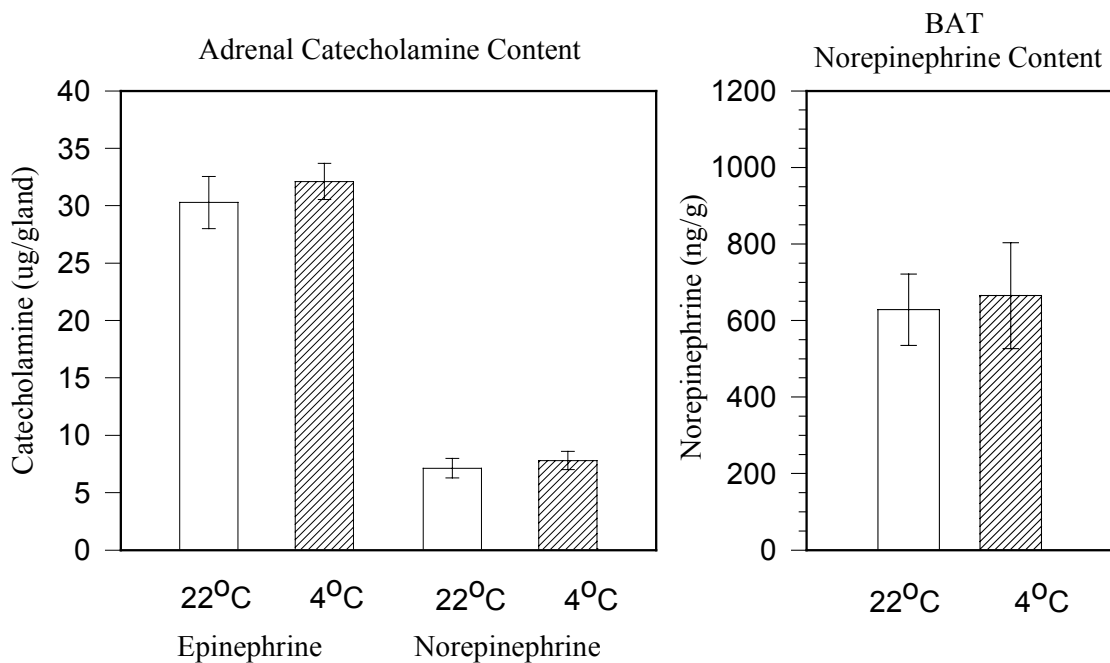


Fig. 13. Effects of propranolol, 3 mg/kg, i.p., on adrenal epinephrine and norepinephrine (NE) content and brown adipose tissue (BAT) NE content in rats kept at 22°C (n=7) and 4°C (n=7). The animals were treated with propranolol and sacrificed 4 hours later. The adrenal and BAT catecholamine content was not different between the two groups.

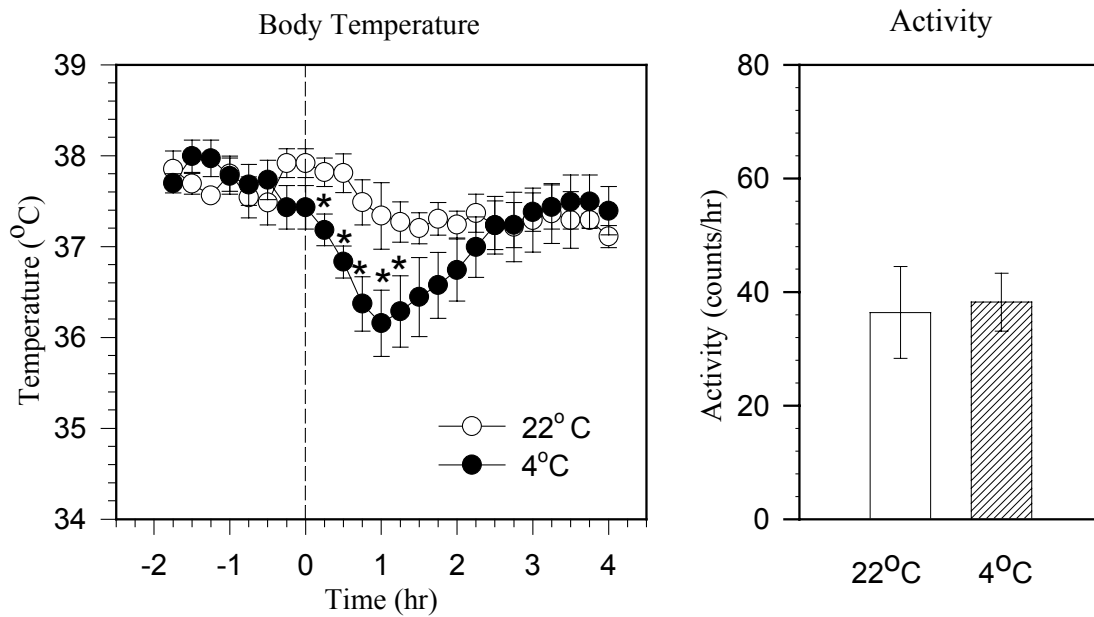


Fig. 14. Effects of phentolamine, 2 mg/kg, i.p. and propranolol, 3 mg/kg, i.p., on body temperature and locomotor activity of rats kept at 22°C (n=7) and 4°C (n=7). The drugs were administered at time “0”. Repeated measures ANOVA indicated that the body temperature of the groups was significantly different, $p < 0.01$. Asterisks indicate that there is a difference between the two groups at specific time points, Bonferoni t-test. The locomotor activity was not different between the two groups.

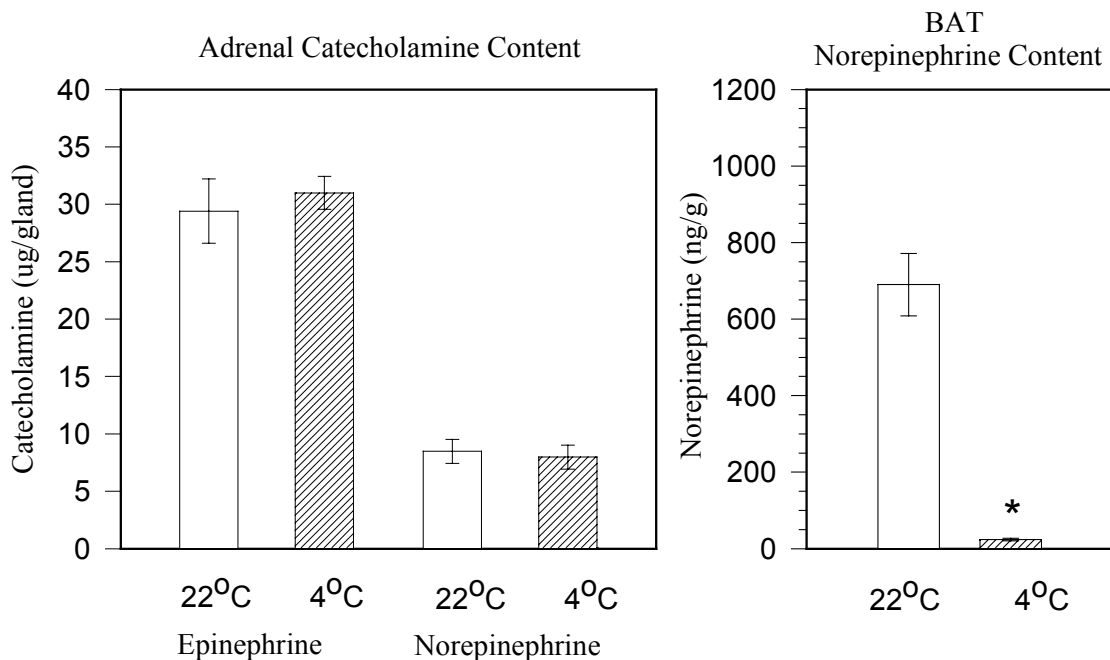


Fig. 15. Effects of phentolamine, 2 mg/kg, i.p. and propranolol, 3 mg/kg, i.p., on adrenal epinephrine and norepinephrine (NE) content and brown adipose tissue (BAT) NE content in rats kept at 22°C (n=7) and 4°C (n=7). The animals were treated with the drugs and sacrificed 4 hours later. The adrenal catecholamine content was not different between the two groups. However, BAT of the cold exposed animals showed a significant depletion of its NE content. Two way ANOVA indicated that the groups are significantly different, $p < 0.001$. An asterisks indicate that there is a significant difference between the phentolamine + propranolol treated group kept at 22°C and the phentolamine + propranolol treated group kept at 4°C, Bonferoni t-test.

Chlorisondamine treatment did not affect adrenal NE or EPI content, Fig. 17, left panel. However, BAT NE content was significantly less (-42%) in the cold exposed chlorisondamine treated animals, $p < 0.001$ (Two-way ANOVA). The BAT NE content of the group of animals treated with chlorisondamine and kept at 22⁰C was significantly different from the group of animals treated with chlorisondamine and kept at 4⁰C (Bonferoni t-test), Fig. 17, right panel.

C. Discussion

The experiments provide quantitative evidence that the sympathetic nervous system mechanisms of heat generation and conservation are essential to the maintenance of body temperature. The pharmacologic blockade of alpha and beta adrenoceptor mediated mechanisms of thermoregulation at 4⁰C resulted in a deficit in body temperature maintenance.

The animals exposed to a cold (4⁰C) ambient temperature for the six-hour observation period could maintain body temperature. Thus, cold exposure alone does not affect body temperature maintenance. In order to determine if the sympathetic nervous system was activated during cold exposure, we measured BAT NE content as an index of sympathetic activity. No change in BAT NE content was observed in animals kept at 4⁰C. While it has been shown by other investigators that BAT is neurally activated during acute cold exposure as evidenced by increased blood flow to BAT (Foster et al., 1978), increased GDP binding to mitochondria of BAT (Trayhurn et al., 1987) and increased BAT NE turnover rate (McDonald et al., 1993), it was found that an increase in neural activity to BAT was not sufficient to change its NE content. However, there are reports

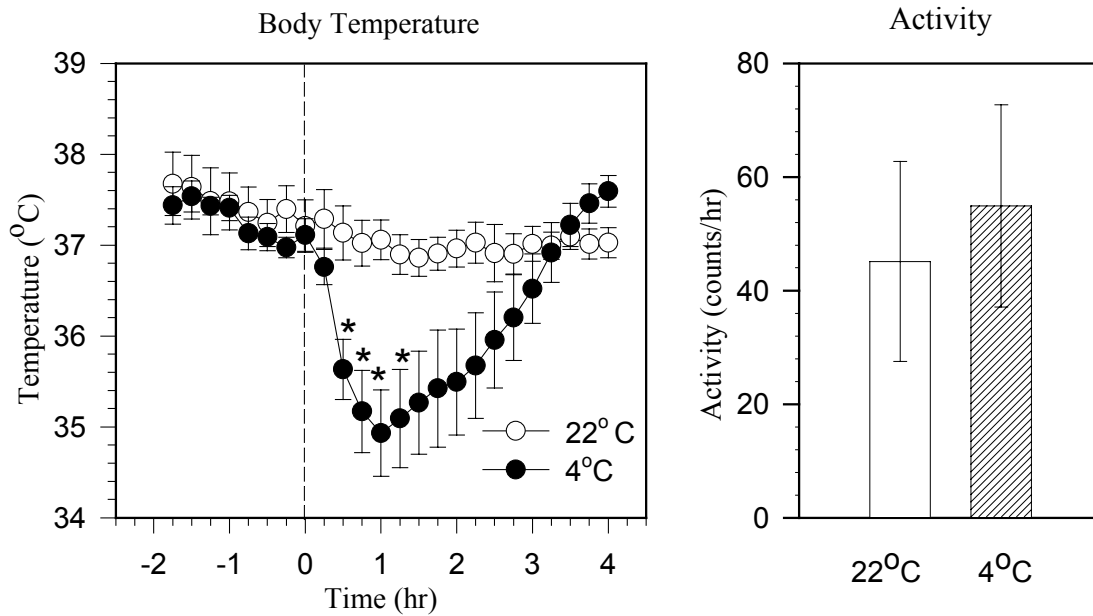


Fig. 16. Effects of chlorisondamine, 3 mg/kg, i.p., on body temperature and locomotor activity of rats kept at 22°C (n=7) and 4°C (n=7). Chlorisondamine was administered at time “0”. Repeated measures ANOVA indicated that the body temperature of the groups was significantly different, $p < 0.01$. Asterisks indicate that there is a difference between the two groups at specific time points, Bonferoni t-test. The locomotor activity was not different between the two groups.

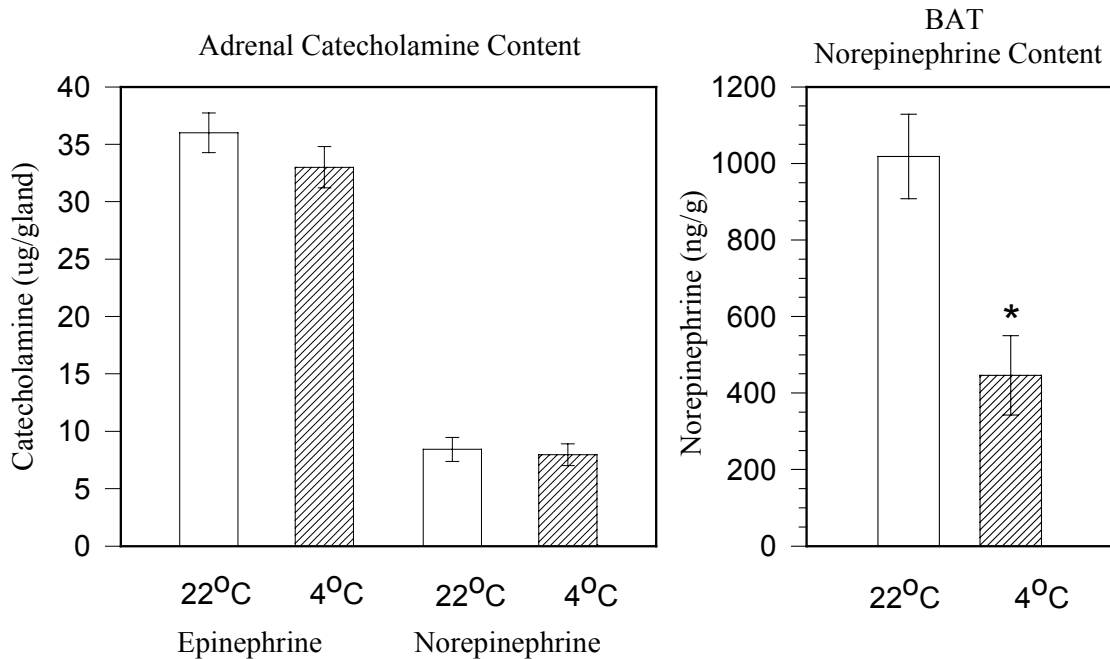


Fig. 17. Effects of chlorisondamine, 3mg/kg, i.p., on adrenal epinephrine and norepinephrine content (NE) and brown adipose tissue (BAT) NE content in rats kept at 22°C (n=7) and 4°C (n=7). The animals were treated with chlorisondamine and sacrificed 4 hours later. The adrenal catecholamine content between the two groups was not different. However, BAT of the cold exposed animals showed a significant depletion of its NE content. Two way ANOVA indicated that the groups are significantly different, $p < 0.001$. An asterisks indicate that there is a significant difference between the chlorisondamine treated group kept at 22°C and the chlorisondamine treated group kept at 4°C, Bonferoni t-test.

that cold exposure alone can cause depletion of BAT NE (King et al., 1999; Brito et al., 1998). The disparity in experimental results is probably best explained by differences in the experimental design of each study, which include variability in the duration of cold exposure and the handling of animals during temperature measurements.

However, the ability to maintain NE content in the presence of increased sympathetic drive to BAT appears to be limited because blockade of alpha-receptors with phentolamine in animals exposed to cold resulted in a decline in BAT NE (-57%). This finding can be interpreted to indicate that phentolamine interfered with alpha-receptor-mediated heat conserving mechanisms, piloerection and cutaneous vasoconstriction, resulting in an augmented activation of sympathetic outflow to BAT. It appears that the activation of BAT offset the effects of phentolamine because core temperature was maintained at normal levels. The prevention of piloerection by phentolamine was visually verified in these studies and the dose of phentolamine used was shown to produce cutaneous vasodilation by others (Lin et al., 1979). Phentolamine did not have an effect on BAT innervation independent of cold exposure because BAT NE content was not diminished in animals kept at 22⁰C.

When beta-receptors were blocked in addition to alpha-receptors, body core temperature significantly declined in cold exposed animals. Thus combined blockade of adrenoceptors presents a greater challenge to thermoregulation than if the alpha- and beta-receptors are independently blocked. This was evidenced by an intense activation of BAT as noted by an almost total disappearance of NE from BAT (-97%).

In rats, there is significant stimulation of heart rate and cardiac output in response to cold exposure (Sun et al., 1997) and the dose of propranolol used in our experiment is sufficient to block these cardiovascular responses mediated via beta-1 receptors (Young et

al., 1992). The extent to which this dose of propranolol would interrupt sympathetic activation of BAT is uncertain because there is substantial evidence to suggest that the functional receptor in BAT is the beta-3 receptor subtype (Zhao et al., 1998). While propranolol is a potent blocker of the beta-1 and beta-2 receptors, higher doses are required to block beta-3 receptor mediated thermogenesis. In one report (Benzi et al., 1988), the dose of propranolol used in the present investigation was shown to block BAT thermogenesis but another report suggests that higher doses are required (Shimizu et al., 1991). Also it must be considered that the endogenous ligand, NE has less affinity for the beta-3 receptor than the beta-1 or beta-2 (Chaudhry et al., 1992; Granneman, 1992; Granneman, 1990). Nevertheless beta-receptor blockade in conjunction with alpha-receptor blockade produced an almost total disappearance of NE from BAT.

Interestingly, the body temperature of animals treated with propranolol while in the cold began to rise and became significantly higher than the animals kept at 22⁰C. One potential explanation for this unexpected finding is that propranolol may interfere with a beta-2 receptor mediated cutaneous vasodilatation that may have been present prior to propranolol administration (Carlisle et al., 1992).

The adrenal content of EPI and NE was measured in these experiments as a potential indicator of adrenal activation but no change in adrenal catecholamines was observed in any of the groups. The absence of any change in content does not rule out the activation of the adrenal since there might be an accelerated synthesis of catecholamines to compensate for the increased secretion. In fact, there is evidence that the adrenals are activated during cold exposure (Himms-Hagan, 1975; Leduc, 1961). One previous study has shown that inhibition of catecholamine synthesis by prior treatment with α -methyl-*p*-tyrosine resulted in a

decreased adrenal NE content when animals were exposed to cold for three hours (Vollmer et al., 1992). In the same study, it was found that a very robust stimulation of the adrenal gland is required before content is measurably reduced. Prolonged cold exposure resulted in a selective depletion of adrenal medullary NE, indicating that the adrenal gland became more active as the duration of cold exposure increased. Another factor that differed from the present investigation was that in the previous study the animals were shaved, thereby increasing the severity of the cold exposure.

The somatic nervous system contributes to thermoregulation through two major actions, shivering induced thermogenesis and through heat generated by increased locomotor activity. The temperature transmitters provided a record of locomotor activity and no increase in voluntary motor activity was detected during cold exposure. Shivering induced heat generation was not measured directly but we visually observed shivering only in the chlorisondamine treated group exposed to cold. This shivering occurred without an increase in locomotor activity. The chlorisondamine group also showed the greatest hypothermic effect during cold exposure. In addition, it was found that the dose of chlorisondamine did not produce a complete blockade of ganglionic transmission because there was a significant depletion of BAT NE content (-42%).

Thus, the experiments in this chapter demonstrate that the integrity of sympathetic input to thermoregulatory effector systems, which include, the adrenals, BAT, cutaneous blood vessels and piloerector smooth muscle is required to maintain body temperature during cold exposure. Blockade of sympathetic neural influences pharmacologically, through the use of alpha- and beta-receptor blockers, demonstrated that both alpha- and beta- adrenoceptor-mediated mechanisms contribute quantitatively to the maintenance of core temperature

during cold exposure. However, due to the overlapping compensations, both alpha- and beta-receptor mechanisms had to be interrupted before a significant deficit in body temperature was detected.

Catecholamine content of BAT and the adrenal gland were measured to determine if the neurotransmitter stores would be reduced proportionately to the intensity of sympathetic nervous system activation. The major finding was that BAT NE content, but not adrenal content, was decreased during cold exposure combined with adrenoceptor blockade. The relationship was consistent with the conclusion that BAT NE content declined when sympathetic neural activity increased to compensate for blockade of adrenoceptor-mediated heat conservation or heat generation mechanisms.

Moreover, this study provided a model to evaluate the effects of fenfluramine on body temperature and BAT activation. This model was used to characterize the effects of fenfluramine on body temperature and sympathetically mediated mechanisms of heat generation and conservation in the following chapters.

Chapter IV. Evaluation of the Effects of Fenfluramine on Sympathetic Nervous System Regulation of Body Temperature

Experiments presented in this chapter were designed to study the effects of fenfluramine treatment on sympathetic nervous system control of thermoregulation using the model developed in chapter III. The results of the experiments in chapter III demonstrated that the sympathetic nervous system plays an important role in the maintenance of body temperature. Pharmacologic blockade of sympathetic nervous system mediated mechanisms of cutaneous vasoconstriction and thermogenesis led to disruption of body temperature homeostasis. In this chapter, experiments were designed to assess the effects of fenfluramine treatment on body temperature maintenance and activation of sympathetic effector systems involved in the maintenance of body temperature that include the BAT, adrenal gland, white adipose tissue and the heart.

As discussed in the literature survey (Chapter I), the effects of fenfluramine on thermoregulation are not fully understood. Although, hypothermia is frequently reported following fenfluramine administration (Cryan et al., 2000; Preston et al., 1990), the effect of fenfluramine treatment on body temperature regulation appears to depend on the environmental temperature at which the animal is maintained at the time of fenfluramine administration. At normal laboratory temperatures of around 22⁰C, some studies report that fenfluramine treatment produced a hypothermic response (Cryan et al., 2000) and others report that there was no change in body temperature of experimental animals to fenfluramine treatment (Sugrue, 1984). The lack of consistency in the results of these studies are due to differences in methodology used to measure body temperature, differences in ambient temperature (20-24⁰C) and the use of different doses of fenfluramine. In contrast, at lower

ambient temperatures, fenfluramine treatment is consistently associated with varying degrees of hypothermia (Malberg et al., 1997; Preston et al., 1990).

Therefore, the first objective of this study was to study the relationship between environmental temperature and the effect of fenfluramine on body temperature. Animals were studied at two ambient temperatures, 22 and 4⁰C. Also, the relationship between the dose of fenfluramine and hypothermia was studied. The doses of fenfluramine were selected based on studies conducted by others in which these doses produced an antiobesity effect (Levitsky et al., 1992). The fact that all animals to be used in this study would have implanted temperature transmitters provided an improvement over prior studies (Sugrue, 1984; Preston et al., 1990) in which animals had to be handled to record core temperature using rectal thermometers, which could lead to handling related stress and increase body temperature (Poole et al., 1977).

The results of the experiments described in chapter III demonstrated that sympathetic activation was important to the homeostatic regulation of body core temperature during cold exposure. Therefore the second objective of these experiments was to determine if a perturbation in sympathetic activation of BAT and other sympathetic effector systems, that include the adrenal glands, heart and white adipose tissue, could account for the hypothermia that has been reported to occur following the administration of fenfluramine. Based on the observation from the experiments presented in the previous chapter and the work of others (Brito et al., 1998; King et al., 1999), BAT NE content was used as an indicator of BAT activation. Previous studies, conducted in our laboratory and others', demonstrated that NE content of the adrenal gland, white adipose tissue and heart was depleted during cold exposure, when the sympathetic nervous system is known to be activated (Vollmer et al.,

1992; Garafalo et al., 1996; Himms-Hagan, 1975). Therefore, NE content of BAT, adrenal glands, heart and white adipose tissue were measured as an index of sympathetic activation.

To test the sensitivity of BAT NE content as an index of sympathetic activity, a preliminary study was conducted. Animals were administered dl-fenfluramine. BAT NE content was found to be profoundly depleted (Experiment 1) in the fenfluramine treated animals. Additional experiments were designed based on the preliminary study. The objectives of the experiments were: First, to determine if the BAT NE depletion to fenfluramine treatment is dose related. Second, to characterize the mechanisms by which fenfluramine produced a depletion of BAT NE content. We determined if fenfluramine-induced BAT NE depletion is mediated indirectly, through activation of the sympathetic nervous system versus a direct effect on the NE stores of BAT (a reserpine-like action). This objective was accomplished by studying the effect of fenfluramine on BAT activation in animals pretreated with and without the ganglionic blocking agent pentolinium, which blocks the sympathetic ganglionic transmission to BAT. We also determined if fenfluramine-induced BAT NE depletion is mediated through release of serotonin. This objective was based on literature reports that fenfluramine mediates most of its pharmacologic effects via serotonin release (Cryan et al., 2000; Lin, 1978). This objective was accomplished by studying the effect of fenfluramine on BAT activation in animals pretreated with and without fluoxetine, which blocks fenfluramine-mediated serotonin release.

A. Protocols

1. Experiment 1. Effects of dl-fenfluramine on body temperature and BAT NE content.

This was a preliminary experiment conducted to determine if fenfluramine produced a change in body temperature and BAT NE content. All animals used in this study were implanted with temperature transmitters. Two groups of animals (n=7/group) were studied at an ambient temperature of 22⁰C and two groups (n=7/group) were studied at an ambient temperature of 4⁰C. Baseline temperature measurements were made for two hours. The animals were then treated with dl-fenfluramine, 10 mg/kg, i.p. or saline and observed for four hours. At the end of the observation period, the animals were anesthetized with pentobarbital, 60 mg/kg, i.p. and tissues were removed for catecholamine content analysis. Intrascapular BAT was removed and frozen for later biochemical analysis. The catecholamine content of BAT was analyzed using HPLC as described in chapter II.

The racemic mixture, dl-fenfluramine was used in this protocol. The dose, 10 mg/kg, of dl-fenfluramine was selected based on studies conducted by other investigators in which the antiobesity effect of fenfluramine was demonstrated at this dose (Preston et al., 1990).

2. Experiment 2. Effects of d-fenfluramine on body temperature and catecholamine content of sympathetically innervated tissues.

This experiment was conducted to determine if the hypothermia and BAT NE depletion produced by fenfluramine was dose-related and if the effect depends on the ambient temperature at which the animals receive fenfluramine. Also, the effects of fenfluramine on the NE content of other sympathetically innervated organs including the adrenal gland, heart and white adipose tissue was determined. The d-isomer of fenfluramine

was used in this study since it affects only the serotonergic neurons whereas the racemic mixture has been shown to affect catecholaminergic neurons as well (Invernizzi et al., 1986). Also, d-fenfluramine is known to be more potent than the racemic mixture. Henceforth, all experiments were conducted using the d-isomer. All animals used in this study were implanted with temperature transmitters. Three groups (n=7/group) were studied at 22°C and three groups (n=7/group) at 4°C. Three treatments were investigated at each temperature, saline, d-fenfluramine, 3 or 10 mg/kg, i.p.

As described in Experiment 1, the experiments were conducted over a six-hour period. Two hours of baseline body temperature measurements were obtained. The animals received treatments and were observed for another four hours. At the end of the observation period, the animals were anesthetized with pentobarbital, 60 mg/kg, i.p. and intrascapular BAT, adrenal glands, heart and perirenal WAT were removed for catecholamine content analysis using HPLC.

The doses of d-fenfluramine used in this experiment were selected based on studies conducted by other investigators in which it was demonstrated that these doses of d-fenfluramine had appetite suppressive and BAT stimulant thermogenic activity (Rothwell et al., 1992; Jackson et al., 1997).

3. Experiment 3. Effects of pentolinium and fluoxetine pretreatment on d-fenfluramine-induced BAT NE depletion.

This experiment was conducted to determine if BAT NE depletion produced by d-fenfluramine was mediated via activation of the sympathetic nervous system and through release of serotonin. This was accomplished by studying the effect of d-fenfluramine on BAT NE content in animals pretreated with and without ganglionic blocker pentolinium and

selective serotonin reuptake inhibitor fluoxetine. The experiments were conducted at 22⁰C since the extent of d-fenfluramine-induced BAT NE depletion was similar in animals maintained at 22 and 4⁰C.

The effects of pretreatment with saline, pentolinium, 10mg/kg, i.p. or fluoxetine, 10 mg/kg, i.p., on d-fenfluramine, 10 mg/kg, i.p.,-induced BAT NE depletion were assessed. The pretreatments were administered after animals were acclimated to the lab conditions for a period of two hours. Thirty minutes after pretreatment, d-fenfluramine, 10 mg/kg, i.p. or saline was administered. After four hours of observation, the animals were anesthetized with pentobarbital, 60 mg/kg, i.p. and intrascapular BAT was removed for NE content analysis. A total of six groups of animals (n=7/group) were studied. Group 1 served as controls and received saline pretreatment followed by a second treatment with saline. Group 2 received saline pretreatment followed by a second treatment with fenfluramine. Group 3 received pentolinium pretreatment followed by a second treatment with saline. Group 4 received pentolinium pretreatment followed by a second treatment with fenfluramine. Group 5 received fluoxetine pretreatment followed by a second treatment with saline. Group 6 received fluoxetine pretreatment followed by a second treatment with fenfluramine.

The dose of d-fenfluramine, 10 mg/kg, i.p., was used since it produced the greatest depletion of BAT NE content in our experiments. The dose of pentolinium used, 10 mg/kg, i.p., was shown to have blocked sympathetic ganglionic transmission in rats (Redfern et al., 1995). The dose of fluoxetine, 10mg/kg, was shown to have blocked fenfluramine-induced release of serotonin in rat brain microdialysis studies (Gundlah et al., 1997).

Statistical Analyses

Results are presented as mean \pm S.E. The differences between groups of animals treated with fenfluramine and saline and maintained at 22 and 4⁰C, for individual parameters (BAT, adrenal, WAT, heart NE content) were assessed using two-way analysis of variance (ANOVA). The differences in BAT NE content between fenfluramine-treated groups pretreated with pentolinium, fluoxetine or saline were assessed using one-way ANOVA. If ANOVA revealed a statistically significant difference, post hoc pairwise comparisons of groups were done using Bonferoni t-test.

The differences between multiple groups (saline, fenfluramine treated groups at 22 and 4⁰C) and between multiple measurements taken over a period of time (-120 to 240 minutes.) were assessed using repeated measures ANOVA. Post-hoc pairwise comparisons were done using Bonferoni t-test. A statistically significant effect was accepted when $p < 0.05$.

B. Results.

1. Experiment 1. Effects of dl- fenfluramine, 10 mg/kg, i.p., on body temperature and BAT NE content.

During the first two hours of the experimental protocol (baseline period), there was no difference in the body temperature between the groups of animals maintained at 22⁰C and 4⁰C. There was no difference in body temperature between the two groups of animals treated with saline and kept at 22 and 4⁰C, Fig. 18.

There was no difference in body temperature between the group of animals treated with dl -fenfluramine and the group of animals treated with saline and kept at 22⁰C. However animals that were treated with dl-fenfluramine at 4⁰C, became significantly

hypothermic, $p < 0.01$ (Repeated measures ANOVA). Post hoc Bonferoni t-test indicated that the body temperature of the animals treated with dl-fenfluramine at 4°C was significantly different from the group of animals treated with saline at 4°C , Fig. 18. BAT NE content of groups of animals that received dl-fenfluramine was significantly depleted (-89.9% at 22°C and -91.3% at 4°C), $p < 0.001$ (Two way ANOVA). Post hoc Bonferoni t-test indicated that the groups of animals that received fenfluramine at 22 and 4°C were significantly different from the respective saline control groups. The extent of BAT NE depletion, produced by fenfluramine in animals maintained at 22 or 4°C , was not different, Fig. 19.

2. Experiment 2. Effects of d-fenfluramine, 3 and 10 mg/kg, i.p., on body temperature and catecholamine content of sympathetically innervated tissues.

During the first two hours of the experimental protocol (baseline period), there was no difference in the body temperature between the groups of animals, Fig. 20, top panel. There was no difference in the body temperature of group of animals treated with d-fenfluramine, 3 mg/kg and the saline-treated control group. However, rats treated with the higher dose of d-fenfluramine, 10mg/kg produced a significant fall in body temperature, $p < 0.01$ (Repeated measures ANOVA). Post hoc Bonferoni t-test indicated that the group of animals treated with fenfluramine, 10 mg/kg, was significantly different from the saline control group, Fig. 20, top panel.

During the first two hours of the experimental protocol (baseline period), there was no difference in the body temperature between the groups of animals maintained at 4°C , Fig. 20, bottom panel. In animals maintained at 4°C , d-fenfluramine produced a significant dose-related decrease in body core temperature, $p < 0.01$ (Repeated measures ANOVA).

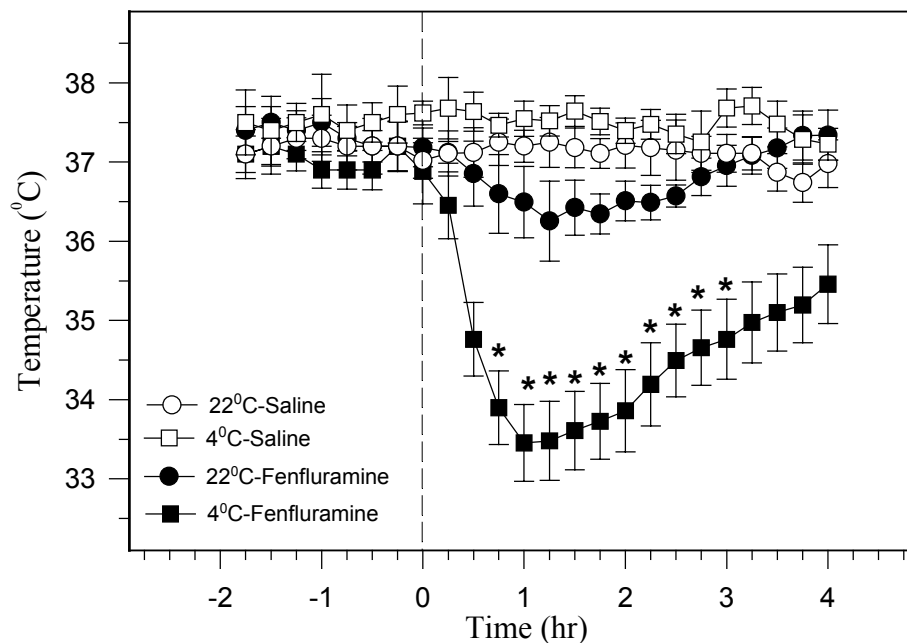


Fig. 18. Effects of saline or dl-fenfluramine , 10 mg/kg, i.p., on body temperature of rats kept at 22 and 4°C (n=7/group). Animals with implanted temperature transmitters were maintained at 22 and 4°C for six hours. Two hours of baseline body temperature data was collected and fenfluramine or saline were administered at time “0”. In animals maintained at 4°C, fenfluramine produced a significant hypothermia. Repeated measures ANOVA indicated that the groups are significantly different, $p < 0.01$. Asterisks indicate a significant difference between the fenfluramine and the saline control group maintained at 4°C at specific time points, Bonferoni t-test.

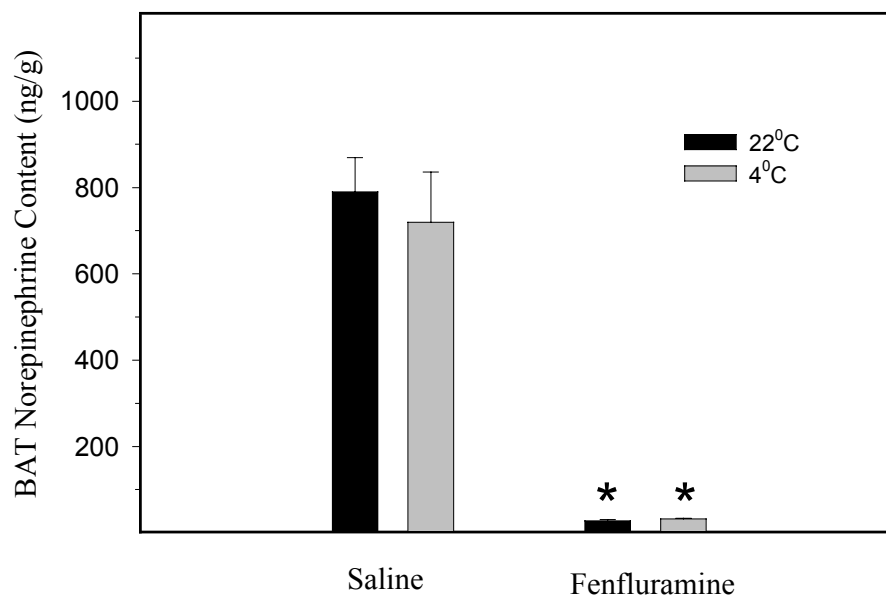


Fig. 19. Effects of saline or dl-fenfluramine, 10 mg/kg, i.p., on brown adipose tissue (BAT) norepinephrine (NE) content of rats kept at 22 and 4°C (n=7/group). The animals were treated with saline or fenfluramine and sacrificed four hours later. Fenfluramine produced a significant depletion of BAT NE content in rats kept at 22 and 4°C. The extent of depletion was similar at 22 and 4°C. Two way ANOVA indicated that the groups are significantly different, $p < 0.001$. Asterisks indicate a significant difference between the fenfluramine and the respective saline control groups, Bonferoni t-test.

Post hoc Bonferoni t-test indicated that the groups of animals treated with d-fenfluramine, 3 and 10 mg/kg were significantly different from the saline control group, Fig. 20, bottom panel.

d-Fenfluramine produced a dose-related decrease in BAT NE content (-57.4% at 3 mg/kg and -75.9% at 10 mg/kg dose), $p < 0.001$ (Two-way ANOVA). The BAT NE content was significantly reduced at 3 and 10 mg/kg doses. Similar decrements in BAT NE content were produced by d-fenfluramine at 22 and 4°C. Post hoc Bonferoni t-test indicated that the BAT NE content of groups of animals treated with d-fenfluramine, 3 and 10 mg/kg, were significantly different from the respective saline control groups. Also, the BAT NE content of the group of animals treated with d-fenfluramine, 3 mg/kg, was significantly different from the group of animals treated with d-fenfluramine, 10 mg/kg, Fig. 21, top panel.

There was no difference in the WAT NE content between the d-fenfluramine and saline treated groups at 22 and 4°C, Fig. 21, bottom panel. Also, there were no differences in the adrenal NE and EPI, heart NE contents between the d-fenfluramine and saline treated groups at 22 and 4°C, Table 1.

3. Experiment 3. Effects of pentolinium, 10 mg/kg, i.p., and fluoxetine, 10 mg/kg, i.p., pretreatment on d-fenfluramine-induced BAT NE depletion.

d-fenfluramine produced a significant decrease in BAT NE content, $p < 0.001$ (One-way ANOVA). Post hoc Bonferoni t-test indicated that the BAT NE content of the group of animals treated with fenfluramine-saline was different from the saline-saline control group. In the group of animals that were pretreated with pentolinium, d-fenfluramine did not produce BAT NE depletion when compared to the group of animals treated with saline and d-fenfluramine. Also, in the group that was pretreated with pentolinium and given saline

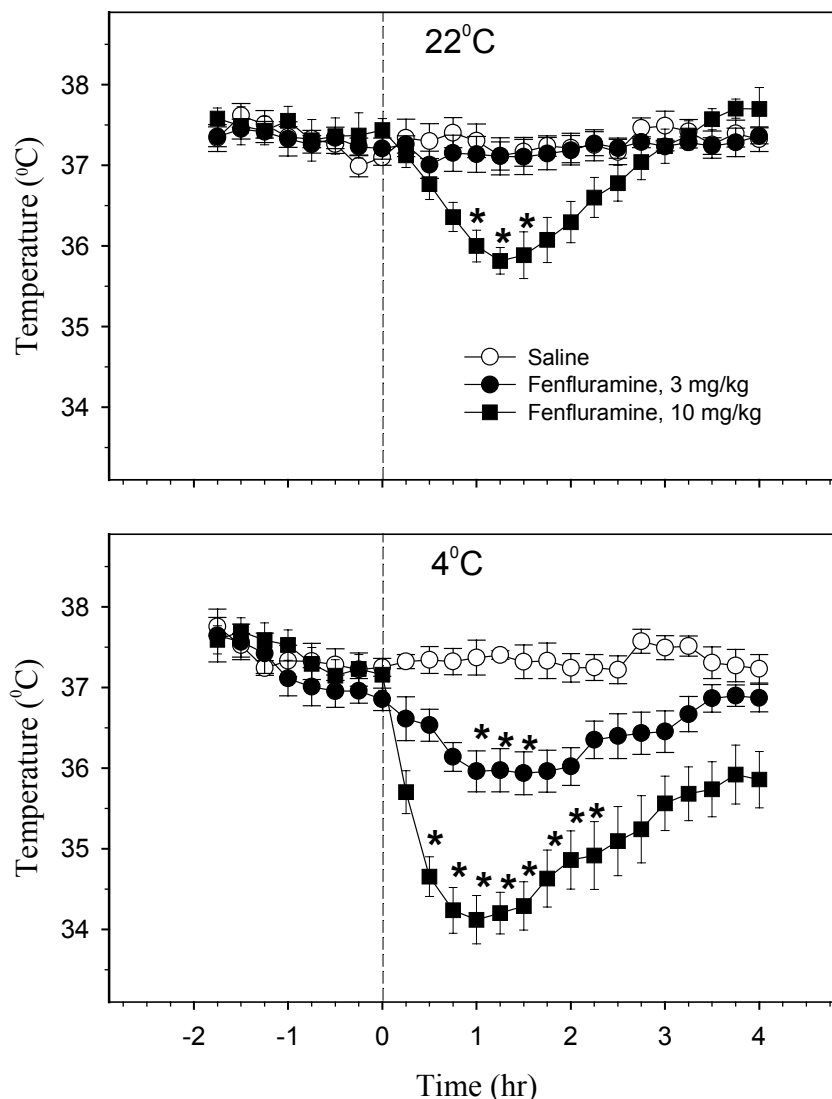


Fig. 20. Effects of saline or d-fenfluramine, 3, 10 mg/kg, i.p., on body temperature of rats kept at 22 and 4°C (n=7/group). Fenfluramine or saline were administered at time “0”. At 22°C, fenfluramine, 10 mg/kg, produced hypothermia whereas the lower dose, 3 mg/kg, had no effect on body temperature. Repeated measures ANOVA indicated that the groups are significantly different, $p < 0.01$. Asterisks indicate a significant difference between the fenfluramine, 10mg/kg and the saline group at specific time-points, Bonferoni t-test. At 4°C, fenfluramine, 3 and 10 mg/kg, produced hypothermia, the higher dose producing a greater hypothermia. Repeated measures ANOVA indicated that the groups are significantly different, $p < 0.01$. Asterisks indicate a significant difference between the fenfluramine, 3 and 10 mg/kg and the saline group at specific time-points.

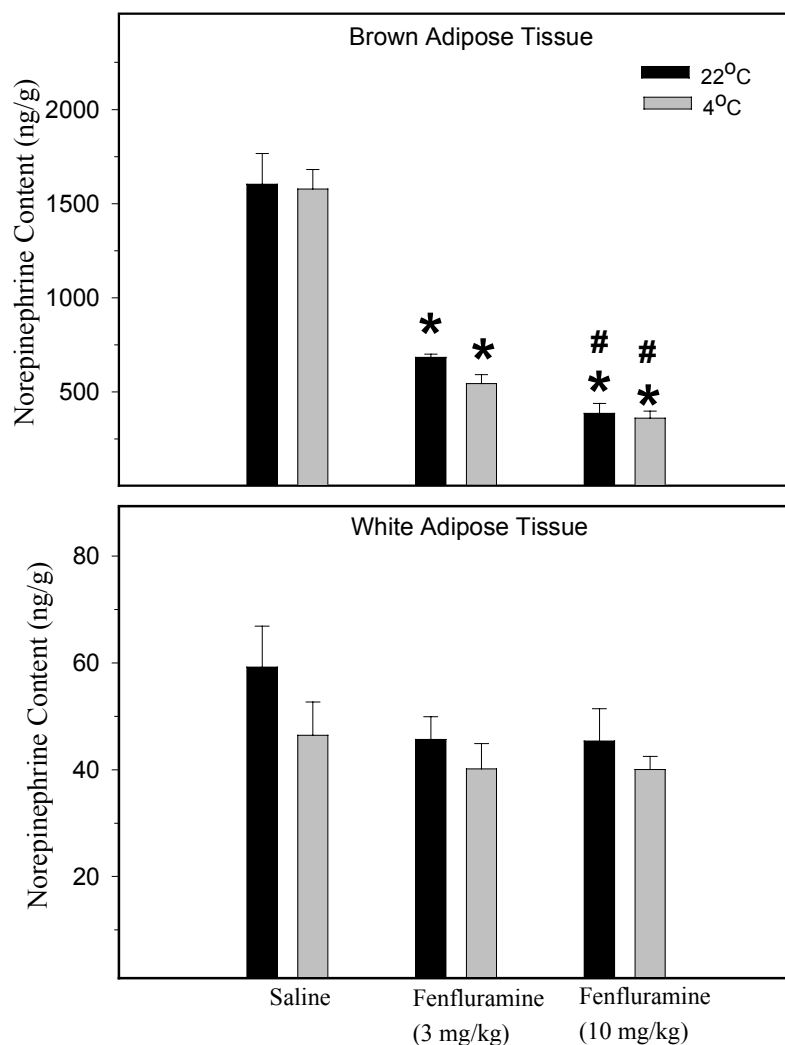


Fig. 21. Effects of saline or d-fenfluramine, 3 and 10 mg/kg, i.p., on brown adipose tissue (BAT) and white adipose tissue (WAT) norepinephrine (NE) content of rats kept at 22°C and 4°C (n=7/group). The animals were treated with fenfluramine or saline and sacrificed four hours later. Fenfluramine, 3 and 10 mg/kg, produced a significant depletion of BAT NE content, the higher dose producing a greater depletion. The extent of depletion was similar at 22 and 4°C. Two way ANOVA indicated that the groups are significantly different, $p < 0.001$. Asterisks indicates a significant difference between the fenfluramine, 3, 10 mg/kg and the saline group, the # sign indicates that the fenfluramine, 3 mg/kg, is different from the fenfluramine, 10 mg/kg, group, Bonferoni t-test. Fenfluramine treatment did not have an effect on WAT NE content.

Table 1. Effects of d-fenfluramine (FEN) on Adrenal and Heart Catecholamine Contents in Rats Kept at 22 and 4⁰C

Tissue	Catecholamine	Ambient Tempera ture (°C)	Treatment		
			Saline	d-FEN (3 mg/kg)	d-FEN (10mg/kg)
Adrenal	Norepinephrine (µg/gland)	22	6.2 ± 0.5	5.9 ± 0.3	5.2 ± 0.5
		4	5.5 ± 0.6	5.2 ± 0.5	5.2 ± 0.6
	Epinephrine (µg/gland)	22	25 ± 1.3	22.7 ± 1.5	22 ± 1.4
		4	24.2 ± 1.3	21.6 ± 2.3	21.1 ± 2.9
Atria	Norepinephrine (µg/g of tissue)	22	1.4 ± 0.4	1.4 ± 0.2	1.4 ± 0.3
		4	1.3 ± 0.3	1.3 ± 0.1	1.4 ± 0.3
Ventricle		22	0.7 ± 0.1	0.7 ± 0.1	0.7 ± 0.1
		4	0.7 ± 0.1	0.7 ± 0.1	0.7 ± 0.1

instead of d-fenfluramine, BAT NE content was not different from the saline-saline control group, Fig. 22. Thus, Pentolinium appeared to produce a more complete ganglionic blockade to BAT compared to chlorisondamine since, in our experiments, chlorisondamine produced a significant depletion in BAT NE content. Therefore, henceforth pentolinium will be used in our studies to produce ganglionic blockade.

In the group of animals that was pretreated with fluoxetine, subsequent fenfluramine treatment did not produce BAT NE depletion when compared to the group of animals treated with saline and d-fenfluramine, Bonferoni t-test. Also, in the group that was pretreated with fluoxetine and given saline instead of d-fenfluramine, BAT NE content was not different from the saline-saline control group, Fig. 23.

C. Discussion

The results of the experiments indicate that fenfluramine administration resulted in hypothermia. The magnitude of hypothermia was related to the ambient temperature at which the animals received fenfluramine. In addition, the magnitude of hypothermia was also directly related to the dose of fenfluramine. Fenfluramine treatment led to a profound depletion of BAT NE content. The depletion of BAT NE by fenfluramine was related to the dose of fenfluramine. However, the depletion was not dependent on the ambient temperature at which the animals received fenfluramine.

dl-fenfluramine, 10 mg/kg, did not affect body temperature maintenance when administered to animals kept at 22⁰C, whereas in animals kept at 4⁰C, the same dose of dl-fenfluramine produced a significant hypothermia. dl-fenfluramine produced a significant depletion of BAT NE content in animals kept at both 22 and 4⁰C.

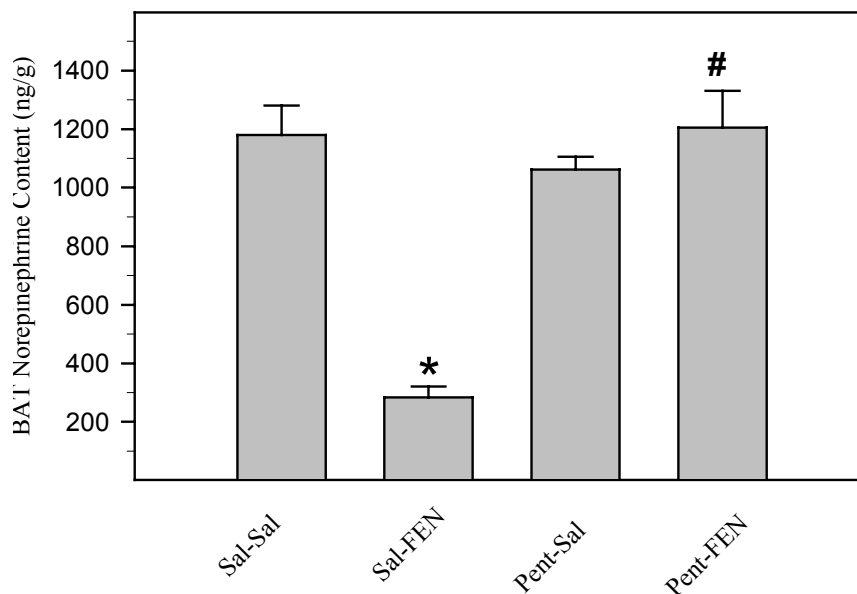


Fig. 22. Effects of saline (Sal) or pentolinium (Pent), 10mg/kg, i.p. pretreatment on d-fenfluramine (FEN), 10 mg/kg, i.p. induced changes in brown adipose tissue (BAT) norepinephrine (NE) content (n=7/group). Two groups of animals were treated with saline and two groups with pentolinium and after 30 minutes, they received saline or fenfluramine. The animals were sacrificed four hours later. Fenfluramine produced a significant depletion of BAT NE content. Petolinium pretreatment prevented fenfluramine-induced BAT NE depletion. Pentolinium treatment did not have any effect on BAT NE content. One way ANOVA indicated that the groups are significantly different, $p < 0.001$. An asterisk indicates that the BAT NE content of the Sal-FEN group is different from that of the Sal-Sal control group. The # sign indicates that the BAT NE content of the Pent-FEN group is different from the Sal-FEN group, Bonferoni t-test.

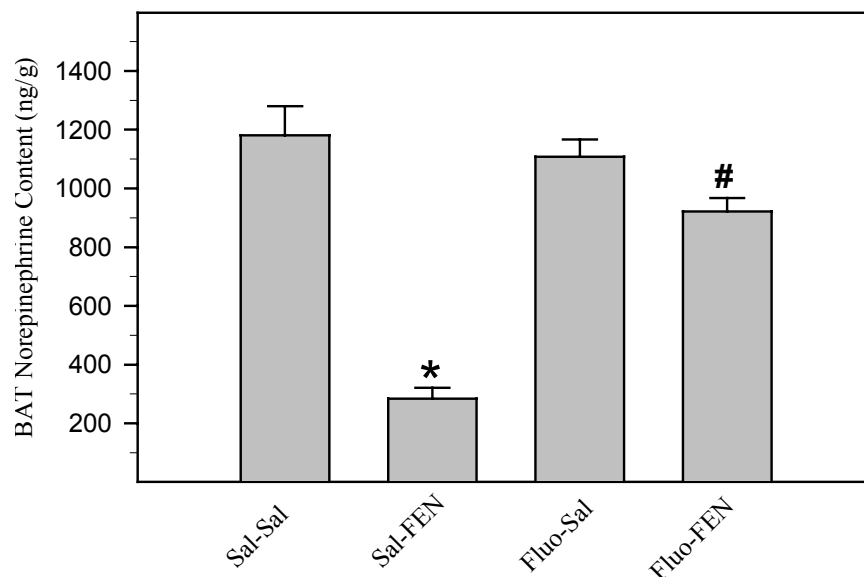


Fig. 23. Effects of saline (Sal) or fluoxetine (Fluo), 10mg/kg, i.p. pretreatment on d-fenfluramine (FEN), 10 mg/kg, i.p. induced changes in brown adipose tissue (BAT) norepinephrine (NE) content (n=7/group). Two groups of animals were treated with saline and two groups with fluoxetine and after 30 minutes, the animals received saline or fenfluramine. The animals were sacrificed four hours later. Fenfluramine produced a significant depletion of BAT NE content. Fluoxetine pretreatment prevented fenfluramine-induced BAT NE depletion. Fluoxetine treatment did not have any effect on BAT NE content. One way ANOVA indicated that the groups are significantly different, $p < 0.001$. An asterisk indicates that the BAT NE content of the Sal-FEN group is different from that of the Sal-Sal control group. The # sign indicates that the BAT NE content of the Fluo-FEN group is different from the Sal-FEN group, Bonferoni t-test.

Thus, it appears that although the effect of fenfluramine on body temperature depends on the ambient temperature at which the animals are kept, the effect of fenfluramine on BAT does not depend on the ambient temperature at which the animals are maintained.

Similarly, when animals kept at 22⁰C were treated with d-fenfluramine, 3 mg/kg, the body temperature was not affected. However, the same dose of d-fenfluramine produced a significant hypothermia in animals kept at 4⁰C. Also, d-fenfluramine, 10 mg/kg, produced a greater hypothermia in animals kept at 4⁰C compared to animals kept at 22⁰C. The hypothermia to fenfluramine was exacerbated when animals were exposed to a cold environment of 4⁰C. d-fenfluramine, 3 and 10 mg/kg doses also produced a significant dose-related decrease in BAT NE content in animals kept at 22 and 4⁰C. However, the extent of BAT NE depletion produced by d-fenfluramine was similar in animals kept at 22 and 4⁰C.

Since fenfluramine produced a distinct hypothermia, one might assume that fenfluramine might have interfered with the sympathetic activation of BAT thermogenesis. However, fenfluramine treatment resulted in a significant depletion of BAT NE content suggesting that fenfluramine activated BAT. That BAT is activated by fenfluramine, has been reported by others (Arase et al., 1988; Lupien et al., 1986). The effect of fenfluramine on BAT has been evaluated at 22 or 28⁰C. The exaggerated hypothermic response to fenfluramine at 4⁰C, led to the suggestion that perhaps fenfluramine did not activate BAT as environmental temperature was reduced (Ma et al., 1991). However, our results demonstrate that BAT NE content was depleted by fenfluramine at 4⁰C.

Interestingly, the racemic mixture dl- fenfluramine, at a dose of 10 mg/kg, produced a greater depletion of BAT NE than 10 mg/kg of the d-isomer. Perhaps this is due to the fact

that the l-isomer is reported to have significant effects on central noradrenergic neurons as well as serotonergic neurons (Invernizzi et al., 1986).

Metabolic production of heat by BAT (non-shivering thermogenesis) is controlled by brain thermoregulatory centers via a sympathetic neuronal pathway (Flaim et al., 1976). BAT thermogenesis is particularly important in rodents as a means of maintaining core body temperature. In cold-exposed animals, BAT activation has been measured by such indices as increased GDP binding to mitochondria (Trayhurn et al., 1987), increased firing of sympathetic neurons (Banet et al., 1978) and increased blood flow to BAT (Foster et al., 1977). Local denervation of the sympathetic neurons entering BAT prevents its activation during cold exposure (Foster et al., 1981).

Like cold exposure, fenfluramine produces marked sympathetic activation of BAT as indicated by increased GDP binding (Lupien et al., 1985) and increased blood flow to BAT (Ma et al., 1991). And like cold-induced BAT activation, the effects of fenfluramine were blocked by surgical denervation (Rothwell et al., 1984) of BAT. The depletion of BAT NE content was used as the indicator of sympathetic activity to BAT and it was confirmed that fenfluramine-induced activation of BAT is mediated via sympathetic neurons. This conclusion is supported by the finding that d-fenfluramine-induced depletion of BAT NE content could be completely prevented by pretreatment with the ganglionic blocker pentolinium.

Noradrenergic neurons innervating BAT seem to be quite sensitive to depletion following an increase in sympathetic discharge. After fenfluramine treatment, the depletion occurred over a rather short time period of four hours. However, NE content of other sympathetically innervated tissues, white fat, the adrenals and the heart, were not affected by

d-fenfluramine. Thus, it is unlikely that fenfluramine depleted NE by a direct effect on the nerve terminals, i.e. a reserpine like action, because all noradrenergically innervated tissue should have been similarly affected.

There are limits to the sensitivity of BAT NE content to detect an activation of BAT. For example, cold exposure alone, which probably increased sympathetic outflow to BAT did not produce a measurable depletion. As mentioned previously, other investigators have shown that GDP binding and blood flow to BAT were increased by cold exposure.

The major pharmacologic effect of fenfluramine is its ability to release serotonin from nerve terminals (Raiteri et al., 1995). Fluoxetine is a selective serotonin reuptake inhibitor (SSRI) that has been shown to block fenfluramine-induced release of serotonin (Gundlach et al., 1997). The release of serotonin by d-fenfluramine appears to be a key step in mediating the increase in sympathetic activation of BAT, since the depletion of NE in BAT was totally abolished by fluoxetine. Interestingly, a recent study has demonstrated that the hypothermia to fenfluramine could also be blocked by pretreating animals with sertraline, another SSRI which prevents fenfluramine-induced serotonin release (Cryan et al., 2000).

Fluoxetine treatment alone produced no change in BAT NE content. This is consistent with other studies in which fluoxetine and other SSRIs were shown not to possess thermogenic activity (Connoley et al., 1999). While fluoxetine would be expected to initially increase the synaptic concentration of endogenously released serotonin in a manner directly proportional to the ongoing activity of serotonin neurons, it appears that the serotonin concentration did not rise high enough to elicit a measurable effect on BAT NE content. In contrast, after fenfluramine treatment, BAT appears to be in a state of constant stimulation and seems to be no longer regulated appropriate to the environmental temperature at which

the animals are maintained. Consistent with this concept is the well documented finding that BAT is activated even in animals maintained at a warm thermoneutral environment of 28⁰C (Preston et al., 1990). Also, as evidenced by BAT NE depletion in the present study, BAT was activated to a marked extent at a normal laboratory temperature of 22⁰C. And there was no evidence of further activation by fenfluramine when the animals were kept in a cold environment because the depletion of BAT NE content was the same at 22 and 4⁰C.

Thus, despite the evidence of marked activation of BAT at 22 and 4⁰C, by fenfluramine, animals became hypothermic. This confirms the findings of other investigators that fenfluramine causes hypothermia at ambient temperatures of 22 and 4⁰C (Cryan et al., 2000; Ma et al., 1991). Also, the hypothermic effect was shown to be dose dependent and exacerbated by lowering the environmental temperature to 4⁰C. However, this exaggerated hypothermic response to fenfluramine at 4⁰C is not due to a reduction in the magnitude of BAT activation. Thus, the hypothermia to fenfluramine at 22 and 4⁰C is not due to the lack of activation of BAT thermogenesis. Also, the results demonstrate that d-fenfluramine-induced BAT NE depletion was mediated through sympathetic activation and through release of serotonin.