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Sympathetic activation by fenfluramine depletes brown adipose tissue norepinephrine content in rats

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Abstract

The antiobesity agent fenfluramine (FEN) has been reported to produce an activation of sympathetic neurons to brown adipose tissue (BAT) resulting in thermogenesis. The present study was conducted to determine if FEN-induced activation of BAT is affected by the ambient temperature at which animals are maintained. Body temperature was determined in conscious male Sprague–Dawley rats using implanted temperature transmitters, and sympathetic activation of BAT was determined by measuring norepinephrine (NE) content. Animals maintained at 22 and 4 °C incurred a significant decline in core body temperature following FEN administration. FEN produced a significant depletion of BAT NE content, and the magnitude of BAT NE depletion was related to the dose of FEN (−57.4% at 3 mg/kg dose and −75.9% at 10 mg/kg dose). However, the extent of BAT NE depletion was equal at 22 and 4 °C. BAT NE depletion by FEN appears to depend on activation of sympathetic neurons because it was prevented by pretreatment with the ganglionic blocker pentolinium (10 mg/kg ip). Furthermore, NE depletion by FEN could be blocked with fluoxetine (10 mg/kg ip) pretreatment, indicating that the action of FEN is mediated through the release of serotonin.

Keywords: Fenfluramine; Brown adipose tissue (BAT); Norepinephrine; Thermoregulation

1. Introduction

Fenfluramine (FEN) is an amphetamine analog that was the most widely prescribed antiobesity agent worldwide before being withdrawn from therapeutic use due to reports of pulmonary hypertension and cardiac valvular lesions (Fishman, 1999; Teramae et al., 2000). Despite the toxicological limitations, FEN serves as a prototype against which newer antiobesity agents are compared.

FEN possesses two well-documented actions that contribute to weight loss, appetite suppression and thermogenic activity (Levitsky and Troiano, 1992; Levitsky et al., 1986; Preston et al., 1990). The thermogenic effect of FEN is accounted for by its ability to increase the sympathetic neural drive to brown adipose tissue (BAT) (Lupien and Bray, 1985; Rothwell and Stock, 1987), a major organ of nonshivering thermogenesis in rodents. FEN-induced activation of BAT was indicated by increased blood flow to BAT (Ma and Preston, 1991) and increased BAT mitochondrial activity (Lupien and Bray, 1985; Lupien et al., 1986). FEN stimulation of BAT is mediated via central sympathetic activation because its effects were blocked when the sympathetic nerves to BAT were severed (Rothwell and Stock, 1987).

Despite the thermogenic activity of FEN, hypothermia is frequently reported following its administration (Cryan et al., 2000; Malberg and Seiden, 1997; Preston et al., 1990). The expression of hypothermia following FEN treatment depends on the environmental temperature at which animals are maintained. If rats are maintained at an ambient temperature of 22 °C, hypothermia is observed and the hypothermia is exacerbated when the ambient temperature is lowered to 4 °C (Preston et al., 1990). The exaggerated hypothermic response to FEN at 4 °C led to the suggestion that perhaps FEN-induced activation of BAT was attenuated as environmental temperature was reduced (Ma and Preston, 1991; Preston et al., 1990). Therefore, the primary objective of our study was to determine if FEN-induced activation of BAT depended on the ambient temperature at which the animals received FEN.

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In order to quantify FEN-induced sympathetic activation of BAT at 22 and 4 °C, we used norepinephrine (NE) content of BAT as an index. In a previous study, we found that BAT NE content is unusually sensitive to depletion following sympathetic activation (Subramanian and Vollmer, 2001b). Thus, we hypothesized that BAT NE content might be useful in assessing activation of BAT by pharmacologic agents like FEN that are known to activate BAT. In fact, in a preliminary report of our work, we demonstrated that FEN administration led to a significant decrease in BAT NE content (Subramanian and Vollmer, 2001a).

Additional objectives of the present study were to determine if BAT NE depletion by FEN is dose related, to determine if FEN-induced depletion of BAT NE content is mediated via activation of the sympathetic nervous system and to determine if the effects of FEN on BAT NE content were mediated through release of serotonin.

2. Methods

2.1. General

Male Sprague–Dawley rats (Hilltop Labs, Scottsdale, PA) weighing 250–275 g on delivery were housed in a room maintained at 22±1 °C with a 12:12-h light–dark cycle with food and water available ad libitum. A 1-week acclimatization period was allowed before surgery was conducted to implant temperature transmitters (Minimitter, Sunriver, OR). All experimental procedures were approved by the University of Pittsburgh Institutional Animal Care and Use Committee.

Animals were anesthetized with pentobarbital sodium (60 mg/kg ip) and temperature transmitters were implanted into the abdominal cavity via a midline incision. Following surgery, the animals were housed individually and a minimum of 5 days was allowed for recovery before experiments were performed.

2.2. Protocol 1: effect of DL-FEN on body core temperature and BAT NE content

This experiment was conducted to determine if FEN produced a change in BAT NE content. Four groups of animals were studied. Two groups of animals were kept at the normal laboratory temperature of 22 °C and the other two groups were moved into a walk-in cold room maintained at 4 °C. The cold room is situated in the laboratory and has a glass door, which allows lighting conditions to be the same for all animals. Baseline temperature measurements were obtained over a period of 2 h. The animals were then treated with saline (1 ml/kg ip) or DL-FEN (10 mg/kg ip) and observed for 4 h. Throughout the experimental period, temperature was recorded at 2-s intervals and averaged every 15 min via a computerized data acquisition system. The dose of DL-FEN was selected based on studies conducted by other investigators in which the thermogenic activity of FEN was demonstrated at this dose (Preston et al., 1990). A time frame of 4 h was chosen because others had found that BAT was activated as early as 3–4 h after FEN administration (Lupien and Bray, 1985; Arase et al., 1989) and also because in a previous study we used the same time frame for BAT NE content determinations following adrenergic antagonists (Subramanian and Vollmer, 2001b).

2.3. Protocol 2: effect of D-FEN on body core temperature and BAT NE content

This experiment was conducted to determine if BAT NE depletion produced by FEN was dose related and if the depletion depends on the ambient temperature at which the animals receive FEN. As described above, the experiments were conducted over a 6-h period. Six groups of animals were studied. Three groups were studied at 22 °C and three groups at 4 °C. Three treatments were investigated at each temperature: saline, 3 mg/kg i.p. D-FEN or 10 mg/kg i.p. D-FEN. The doses of D-FEN were selected based on studies conducted by other investigators in which it was demonstrated that D-FEN had appetite suppressive and thermogenic activity (Rothwell and LeFeuvre, 1992).

2.4. Protocol 3: effect of pentolinium and fluoxetine pretreatment on FEN-induced BAT NE depletion

This experiment was conducted to determine if the BAT NE depletion produced by FEN was mediated via activation of the sympathetic nervous system and through release of serotonin. The experiments were conducted at 22 °C. The effects of pretreatment with saline, pentolinium (10 mg/kg ip) or fluoxetine (10 mg/kg ip) on FEN-induced BAT NE depletion were assessed. The pretreatment was administered after animals were acclimated to the lab conditions for a period of 2 h. Thirty minutes after pretreatment, D-FEN (10 mg/kg ip) or saline was administered. After 4 h of observation, the animals were sacrificed and their intrascapular BAT was removed and frozen for further biochemical analysis. A total of six groups of animals 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 FEN. Group 3 received pentolinium pretreatment followed by a second treatment with saline. Group 4 received pentolinium pretreatment followed by a second treatment with FEN. Group 5 received fluoxetine pretreatment followed by a second treatment with saline. Group 6 received flouxetine pretreatment followed by a second treatment with FEN. The dose of pentolinium (10 mg/kg ip) used was shown to have blocked sympathetic ganglionic transmission in rats (Redfern et al., 1995). The dose of fluoxetine (10 mg/kg) used blocked

FEN-induced release of serotonin in rat brain microdialysis studies (Gundlah et al., 1997).

2.5. Analysis of intrascapular BAT, adrenal glands, heart and white adipose tissue (WAT) for determination of catecholamines

At the end of the experiments, the animals were sacrificed and intrascapular BAT, adrenal glands, heart and WAT were removed and frozen (−70 °C) for later analysis of catecholamines. Preceding analyses, the tissues were thawed and homogenized in a solution of 0.1 N perchloric acid containing EDTA and sodium metabisulfite. The samples were centrifuged and the supernatant was stored at −70 °C until assayed. Catecholamines were assayed using high-performance liquid chromatography with electrochemical detection (Waters, Marlborough, MA) (Weicker et al., 1984). After a 1:100 dilution with perchloric acid, adrenal supernatants were directly injected into the HPLC system.

Dihydroxybenzylamine (DHBA) was added to BAT, heart and WAT supernatants as an internal standard and the catecholamines were eluted from alumina. The recovery of catecholamines after extraction was between 65% and 70%. Catecholamines were separated on a 5 μm size 3.9×150 mm C-18 reverse-phase column (Waters). 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 epinephrine was 50 pg/ml.

2.6. Statistical analysis

Results are presented as mean±S.E. The differences between multiple groups maintained at 22 and 4 °C, for individual parameters (BAT, adrenal, WAT and heart NE content) were assessed using two-way analysis of variance. The differences in BAT NE content between D-FEN-treated groups pretreated with pentolinium, fluoxetine or saline were assessed using one-way analysis of variance. If analysis of variance revealed a statistically significant difference, post hoc pairwise comparisons of groups were done using Bonferroni *t* test (SigmaStat, Jandel, San Rafael, CA). The differences between multiple groups (saline, FEN-treated groups at 22 and 4 °C) and between multiple measurements taken over a period of time (−120 to 240 min) were assessed using repeated-measures analysis of variance. Post hoc pairwise comparisons were done using Bonferroni *t* test. A statistically significant effect was accepted when $P<.05$.

3. Results

At the commencement of the experiment, the mean weight of the animals was 352±3.6 g (n=112).

3.1. Protocol 1: effects of DL-FEN on body temperature and BAT NE content

Placing animals in the 4 °C environment produced no change in body temperature when contrasted with animals kept at 22 °C during the 2-h baseline observation period. Furthermore, treatment with saline at 22 or 4 °C produced no difference in body temperature throughout an additional 4-h period (Fig. 1, top panel).

DL-FEN (10 mg/kg) at 22 °C produced no change in body temperature. However, at 4 °C, animals that received DL-FEN became significantly hypothermic ($P<.01$) (Fig. 1, top panel).

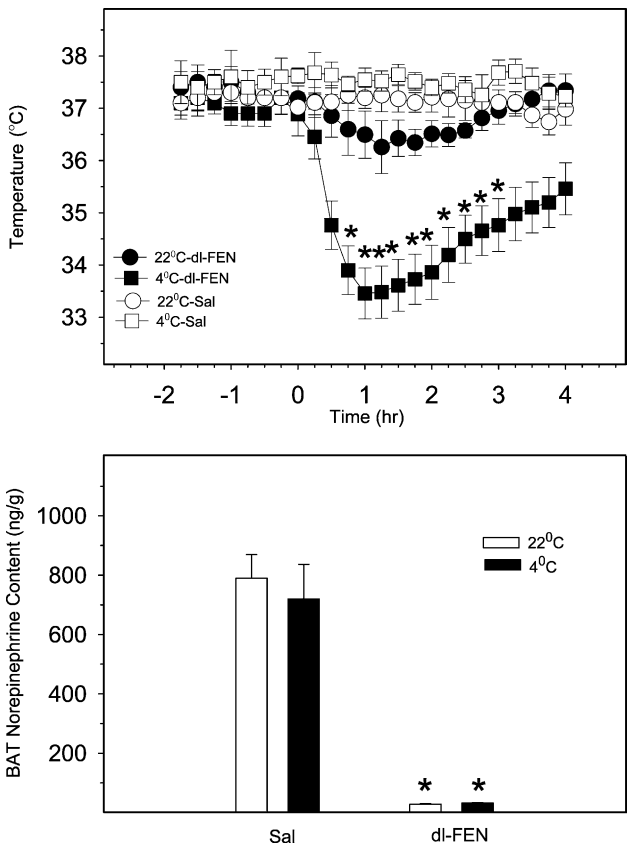

Fig. 1. Top panel: Effect of saline (Sal) or DL-FEN (10 mg/kg ip) administered at time "0" on body temperature of rats kept at 22 and 4 °C (n=6–7/group). Repeated-measures analysis of variance indicated that the groups are significantly different ($P<.01$). Asterisks indicate a significant difference between the DL-FEN and the Sal group at that time point (Bonferroni *t* test). Bottom panel: Effect of Sal or DL-FEN (10 mg/kg ip) on BAT NE content of rats kept at 22 and 4 °C (n=6–7/group). One-way analysis of variance indicated that the groups are significantly different ($P<.0001$). Asterisks indicate a significant difference between the DL-FEN and the Sal group (Bonferroni *t* test).

The two groups of animals that received DL-FEN showed a profound depletion of BAT NE content that was similar in animals kept at 22 and 4 °C ($P<.0001$) (Fig. 1, bottom panel).

3.2. Protocol 2: effects of D-FEN treatment on body temperature, BAT, adrenal, heart and WAT NE content

As was the case in Protocol 1, no changes in body temperature were produced in the two groups of animals treated with saline and maintained at 22 and 4 °C. In rats kept at 22 °C, D-FEN (3 mg/kg) produced no change in body core temperature. However, in rats treated with the higher dose of D-FEN (10 mg/kg), a significant hypothermia was observed ($P<.01$) (Fig. 2, top panel).

D-FEN at 3 and 10 mg/kg doses produced a significant hypothermia in the animals maintained at 4 °C ($P<.01$) and this effect was dose related (Fig. 2, bottom panel). In addition, a dose-related decrease in BAT NE content was produced ($P<.0001$) (Fig. 3, top panel). The magnitude of the decrements in BAT NE content produced by D-FEN at 22 and 4 °C was not different. There was no difference in the WAT NE content between the groups at 22 and 4 °C (Fig. 3, bottom panel).

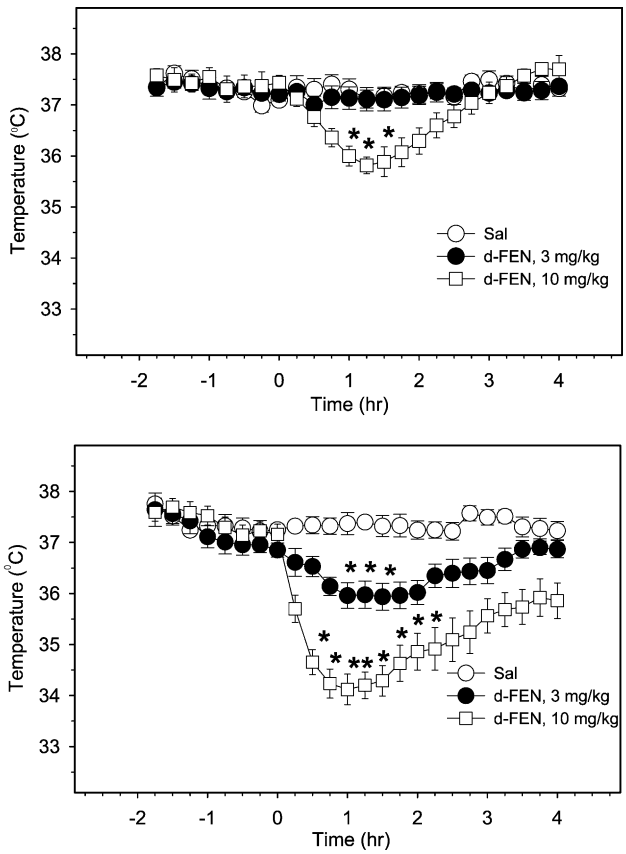

Fig. 2. Top panel: Effect of saline (Sal) or D-FEN (3 and 10 mg/kg ip) administered at time "0" on body temperature of rats kept at 22 °C (n=7/group). Repeated-measures analysis of variance indicated that the groups are significantly different ($P<.01$). Asterisks indicate a significant difference between the D-FEN (10 mg/kg) and the Sal group at a particular time point (Bonferroni t test). Bottom panel: Effect of Sal or D-FEN (3 and 10 mg/kg ip) administered at time "0" on body temperature of rats kept at 4 °C (n=7/group). Repeated-measures analysis of variance indicated that the groups are significantly different ($P<.01$). Asterisks indicate a significant difference between the D-FEN (3 and 10 mg/kg) and the Sal group (Bonferroni t test).

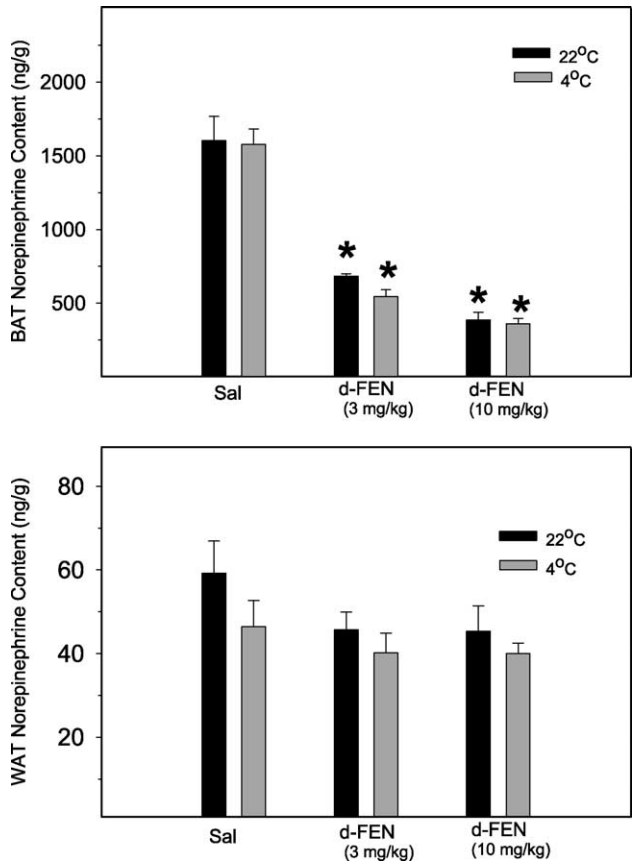

Fig. 3. Top panel: Effect of saline (Sal) or D-FEN (3 and 10 mg/kg ip) on BAT NE content of rats kept at 22 and 4 °C (n=7/group). Two-way analysis of variance indicated that the groups are significantly different ($P<.0001$). Asterisks indicates a significant difference between the D-FEN (3 and 10 mg/kg) and the Sal group (Bonferroni t test). Bottom panel: Effect of Sal or D-FEN (3 and 10 mg/kg ip) on WAT NE of rats kept at 22 and 4 °C (n=7/group). Two-way analysis of variance indicated that the groups are not significantly different.

In addition, there were no differences in the adrenal catecholamine (NE and epinephrine) and heart NE contents among the groups at 22 and 4 °C (Table 1).

3.3. Protocol 3: effect of pentolinium and fluoxetine pretreatment on D-FEN-induced BAT NE depletion in rats

As was the case in Protocol 2, D-FEN produced a significant decrease in BAT NE content as compared to the saline group ($P<.001$). However, pentolinium pretreatment blocked FEN-mediated BAT NE depletion. Pentolinium treatment did not alter BAT NE content (Fig. 4, top panel). Fluoxetine pretreatment also blocked FEN-induced

Table 1
Effect of D-FEN on adrenal and heart catecholamine contents in rats kept at 22 and 4 °C

Tissue	Catecholamine	Ambient temperature (°C)	Treatment		
			Saline	D-FEN (3 mg/kg)	D-FEN (10 mg/kg)
Adrenal	NE (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	NE (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	NE (μg/g of tissue)	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

BAT NE depletion and fluoxetine did not have an effect on BAT NE content (Fig. 4, bottom panel).

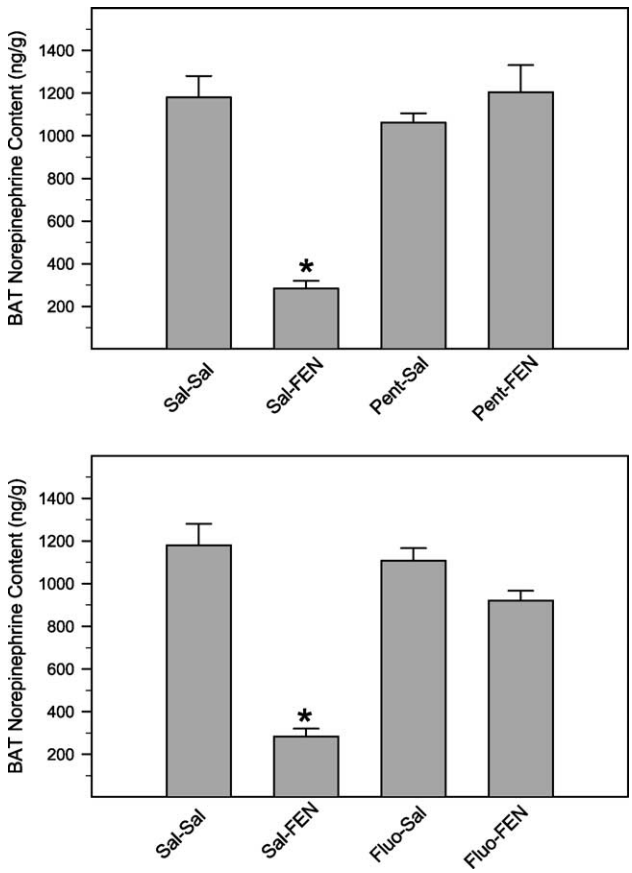

Fig. 4. Top panel: Effect of saline (Sal) or pentolinium (Pent) (10 mg/kg ip) pretreatment on D-FEN (10 mg/kg ip)-induced changes in BAT NE content (n=7/group). One-way analysis of variance indicated that the groups are significantly different (P<.001). The Pent-pretreated group that received D-FEN is significantly different from the Sal-pretreated group that received D-FEN (Bonferroni t test). Bottom panel: Effect of Sal or fluoxetine (Fluo) (10 mg/kg ip) pretreatment on D-FEN (10 mg/kg ip)-induced changes in BAT NE content (n=7/group). One-way analysis of variance indicated that the groups are significantly different (P<.001). The Fluo-pretreated group that received D-FEN is significantly different from the Sal-pretreated group that received D-FEN (Bonferroni t test).

4. Discussion

A novel finding of this study was the observation that FEN produced a marked depletion of NE from BAT that appears to be the result of an increase in the activity of the sympathetic neurons innervating BAT. The extent of BAT NE depletion was equal at 22 and 4 °C. The NE depletion produced by FEN could be prevented by disrupting ganglionic transmission or by blocking the serotonin reuptake transporter. However, despite the fact that FEN produced a marked activation of BAT, animals became hypothermic at a normal laboratory temperature of 22 °C. Moreover, the hypothermia was exacerbated when animals were exposed to a cold environment of 4 °C.

Metabolic production of heat by BAT (nonshivering thermogenesis) is controlled by brain thermoregulatory centers via a sympathetic neuronal pathway (Flaim and Horwitz, 1976; Himms-Hagan, 1991). 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 (Arase et al., 1988) and increased blood flow (Ma and Preston, 1991; Foster and Frydman, 1978) to BAT. Denervation of the tissue prevents its activation during cold exposure (Foster et al., 1981).

Like cold exposure, FEN produces marked sympathetic activation of BAT as indicated by increased GDP binding (Lupien et al., 1986) and increased blood flow to BAT (Ma and Preston, 1991). And like cold-induced BAT activation, the effects of FEN were blocked by surgical denervation (Rothwell and LeFeuvre, 1992). In the present study, we used depletion of BAT NE content as the indicator of sympathetic activity and we confirm that FEN-induced activation of BAT is mediated via sympathetic neurons. This conclusion is supported by the finding that FEN-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 FEN treatment, the depletion occurred over a rather short time period of 4 h. However, NE content of other sympathetically innervated tissues, white fat and the heart, were not affected by FEN. Thus, it is unlikely that FEN 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.

The ability to deplete BAT NE content by activating sympathetic neurons is not unique to FEN. We have previously demonstrated that animals exposed to a cold environment and treated with the α-adrenoceptor antagonist phentolamine showed a marked depletion of BAT NE content (Subramanian and Vollmer, 2001b). We interpreted this finding to indicate that animals with α-receptors blocked were not able to conserve heat by constricting the cutaneous circulation. Therefore, the thermoregulatory control systems produced a greater stimulation of BAT in order to generate heat. As a consequence, NE content of BAT was depleted.

There are limits to the sensitivity of BAT NE content to detect an activation of BAT. For example, in the present investigation, 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 (Trayhurn et al., 1987; Foster and Frydman, 1978).

The major pharmacologic effect of FEN is its ability to release serotonin from nerve terminals (Garattini et al., 1975; Raiteri and Vallebuona, 1995). Fluoxetine is a selective serotonin reuptake inhibitor (SSRI) that has been shown to block FEN-induced release of serotonin (Sabol et al., 1992; Gundlah et al., 1997; Berger et al., 1992). The release of serotonin by FEN appears to be a key step in mediating the increase in sympathetic activation of BAT because the depletion of NE in BAT was totally abolished by fluoxetine.

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 (Gundlah et al., 1997), it appears that the serotonin concentration did not rise high enough to elicit a measurable effect on BAT NE . In contrast, after FEN 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 (Ma and Preston, 1991). Also, as evidenced by BAT NE depletion in the present study, BAT was activated to a marked extent at a normal laboratory temperature of 22 °C. In addition, there was no evidence of further activation by FEN when the animals were kept in a cold environment because the depletion of BAT NE content was the same at 22 and 4 °C. One might suspect that ceiling depletion could have been achieved at 22 °C and so no further depletion was observed when animals were placed at 4 °C. However, we know that the ceiling effect was not reached, because the depletion to 10 mg/kg dose of FEN was significantly higher than the 3 mg/kg dose. Therefore, we know that even though greater BAT NE depletion could be achieved (as shown by the 10 mg/kg dose), the 3 mg/kg dose produced the same degree of depletion at 22 and 4 °C.

The present experiments provide no direct insight into the central location at which FEN is acting. The neural pathways to BAT, as determined by functional studies (Lupien et al., 1986; Amir, 1990; Amir and De Blasio, 1991) and a recent pseudorabies virus tracing study (Bamshad et al., 1999), involve multiple central nervous system sites that include the hypothalamus, raphe nuclei and medullary centers. Serotonergic neurons are anatomically associated with all of these areas. It has been recently demonstrated that the premotor neurons to BAT are located in the raphe pallidus (Morrison et al., 1999) and are under tonic inhibitory influence by GABAergic neurons. Pharmacologic blockade of GABA receptors has been shown to produce a significant increase in postganglionic sympathetic neuronal firing in neurons innervating BAT. The increased neuronal firing was attenuated by 5-HT_{1A} receptor agonist 8-OH-DPAT, which inhibits release of serotonin by acting on presynaptic autoreceptors. Our findings of increased sympathetic activity to BAT by FEN is consistent with these observations because elevated synaptic serotonin concentrations following FEN treatment would be expected to activate the premotor neurons to BAT.

Despite the evidence of marked activation of BAT at 22 and 4 °C by FEN, animals became hypothermic. This confirms the findings of other investigators that FEN causes hypothermia at ambient temperatures of 22 and 4 °C (Cryan et al., 2000; Malberg and Seiden, 1997; Farfel et al., 1995). In the present study, the hypothermic effect was shown to be dose dependent and exacerbated by lowering the environmental temperature to 4 °C. However, this exaggerated hypothermic response to FEN at 4 °C is not due to a reduction in the magnitude of BAT activation. Recently, it was demonstrated that the ability of FEN to produce a hypothermic response is dependent on its uptake into serotonin neurons because sertraline, a SSRI, was shown to block FEN-induced hypothermia in rats (Cryan et al., 2000). However, the mechanism by which FEN elicits hypothermia remains to be elucidated. In a recent study, we reported that FEN produced a significant increase in cutaneous blood flow, which suggests that FEN promotes heat loss (Vollmer et al., 2001).

In conclusion, we demonstrate that D-FEN activates BAT, as shown by decreases in NE content, at 22 and 4 °C. The decrements in NE content were similar at both ambient temperatures, implying that D-FEN activates BAT ther-

mogenesis to the same level at a cooler ambient temperature. The ganglionic blocker pentolinium and the SSRI fluoxetine reversed the effects of D-FEN on BAT NE content, indicating that the effects of D-FEN on BAT are mediated by central sympathetic activation and require the release of central serotonin. We also demonstrate that D-FEN induced a consistent reduction in core temperature, which confirms the results obtained by other investigators.

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