Long-term cigarette smoke exposure increases uncoupling protein expression but reduces energy intake

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Abstract

The appetite suppressing effect of tobacco is a major driver of smoking behaviour; however few studies have addressed the effects of chronic cigarette smoke exposure (SE) on appetite, body weight and metabolic markers. We compared the effects of SE to equivalent food restriction (pair-fed, PF), against sham-exposure, on body weight, adiposity, cytokines, and levels of uncoupling proteins (UCP) and brain neuropeptide Y (NPY) in male Balb/c mice. SE rapidly induced anorexia, and after 12 weeks, SE and PF groups were lighter than control animals (23.9 ± 0.2, 25.5 ± 0.5, 26.8 ± 0.4g respectively, $P < 0.05$). White fat (WAT) masses were reduced by both SE and PF. Plasma leptin and insulin were reduced in SE mice; insulin was further reduced by PF. Brown fat UCP1 and 3 mRNA were increased in SE animals relative to PF animals, possibly promoting thermogenesis. WAT mRNA expression of the inflammatory cytokine, TNFα was doubled by SE, while IL-6 was reduced by both PF and SE. Hypothalamic NPY content was increased by SE (89.3±2.8 vs. 75.9±2.4 ng control, $P < 0.05$), and more in PF mice (100.7±3.4 ng, $P < 0.05$ compared to both groups), suggesting disinhibition due to reduced adipose-derived leptin.

In contrast to equivalent food restriction, cigarette smoke exposure reduced body weight and total hypothalamic NPY, and increased thermogenesis and markers of inflammation. The suppressed hypothalamic NPY and increased UCPs may contribute to the spontaneous hypophagia and extra weight loss in SE animals. These findings contribute to our understanding of weight loss in smoking-related lung disease, suggesting a greater impact than that due to anorexia alone.

Section: 6 Regulatory Systems

Key words: NPY, ATGL, UCPs, TNFα, wasting

1. Introduction

Smoking is an addictive behaviour with a low cessation rate, and relapse is common after attempts to quit (Han et al., 2006). Nicotine is primarily responsible for the highly addictive properties of cigarettes. The appetite suppressing effect of tobacco is another major driver of smoking behaviour, and potential weight gain on cessation may prevent people from quitting (Richmond et al., 1993). Many studies have investigated the mechanisms underlying the anorexia and weight loss accompanying smoking by administering various doses of nicotine (Frankham and Cabanac, 2003; Frankish et al., 1995; Li et al., 2000); however few have addressed the effects of smoke. We have previously used short term (4 days) and subchronic (4 weeks) smoke exposure to investigate how smoking itself directly affects central feeding regulation and metabolic regulation (Chen et al., 2005; Chen et al., 2006).

Despite considerable daily variation in both energy intake and expenditure, most animals, including humans, maintain a steady body weight in the long term. A state of energy balance exists, when total body energy expenditure is equal to the energy substrates absorbed from the diet. In mice exposed to either short-term or sub-chronic cigarette smoke we have demonstrated that this balance is significantly disturbed (Chen et al., 2005; 2006). Smoke-exposed (SE) animals had lower energy intake compared with sham exposed animals. However, after 4 weeks of intervention downregulation of peripheral markers of energy expenditure were less affected by smoke exposure than by equivalent food restriction in the face of similar body weight, suggesting additional metabolic effects of cigarette smoke exposure to its impact on appetite (Chen et al., 2006). Furthermore, after 4 weeks of smoke exposure treatment fat loss contributed to the reduced weight gain in smoke-exposed animals, which was not observed in animals with equivalent food restriction, suggesting a divergence of substrate usage during smoke exposure and food restriction alone. Additionally,

significantly downregulated adipose triglyceride lipase (ATGL) expression at 4 weeks suggests a reduced usage of lipid store (Chen et al., 2006); however, this adaption was only due to reduced energy intake caused by smoke exposure.

Our work has also implicated central dysregulation of hypothalamic appetite regulation in this process. The hypothalamus is a critical integrator and processor of peripheral metabolic information to maintain energy balance. The paraventricular nucleus (PVN), in the anterior hypothalamus, is richly supplied by axons projecting from the arcuate nucleus (Arc) (Cowley et al., 1999; Sawchenko and Swanson, 1983). The Arc is rich in terminals containing neurotransmitters known to regulate appetite, such as neuropeptide Y (NPY) (Cowley et al., 1999). NPY is a powerful neurochemical stimulator of feeding in many species (Hansen et al., 2004; Raposinho et al., 2001). Hypothalamic NPY levels reflect overall nutritional status, contributing to the long-term regulation of energy homeostasis. The PVN is the only hypothalamic nucleus in which release of NPY was augmented both *in vivo* and *in vitro* in response to fasting and before initiation of feeding (Dube et al., 1992; Kalra et al., 1991). In sub-chronic cigarette smoke exposure (4 weeks), the inhibition of NPY production in the PVN may contribute to the spontaneously reduced caloric intake (Chen et al., 2006).

However, the physiological changes described above occurred in response to a short-term intervention relative to the life span of a mouse; whereas cigarette smoking is a long-term behaviour. Studies of long-term smoke exposure are needed to more accurately mimic changes known to occur in human long-term smokers. The body may adapt differently to cope with a long period of negative energy balance to enable survival, such as increasing lipolysis of adipose tissue, or markedly reducing energy expenditure. It is also unknown

whether long-term mild food restriction will diminish the appetite inhibitory effects of cigarette smoke. These questions formed the rationale of the current work.

2. Results

Daily energy intake before treatment was similar between groups, and this was reduced by 16% by cigarette smoke exposure ($P < 0.05$ compared to pre-exposure intake, Table 1). Sham-exposed mice gained weight over the experimental period, increasing body weight by 23%. One week after the commencement of smoke exposure, the body weight of the SE group was significantly lower than the control group ($P < 0.05$, Fig.1), thereafter body weight gain of the SE group remained stable until 8 weeks. At the conclusion of the experiment, the average weight gain of the SE group was only 2.63 (± 0.24) g compared to 5.50 (± 0.23) g in the control group ($P < 0.05$). The body weight trajectory of the PF group lay between control and SE groups, becoming lower than the control group after 2 weeks. After 12 weeks of pair feeding, the body weight of the PF mice was significantly greater than SE animals ($P < 0.05$, Fig. 1), and the weight gain of the PF mice (3.51 ± 0.35 g) was also intermediate ($P < 0.05$, compared to both control and SE groups, Fig. 1).

The weights of kidney, retroperitoneal (Rp) white adipose tissue (WAT) and testicular WAT were reduced to similar levels by smoke exposure and equivalent food restriction ($P < 0.05$ compared with control, Table 1), while liver and brown adipose tissue (BAT) mass was only reduced by smoke exposure ($P < 0.05$ compared with control and PF groups). When data were adjusted for body weight, WAT weights remained similar in both SE and PF groups (RpWAT: 0.55 ± 0.02% in SE and 0.49 ± 0.04% in PF group vs. 0.72 ± 0.03% in control group, overall $P < 0.01$; $P < 0.05$, SE and PF groups vs. control group; testicular WAT: 1.89 ± 0.10% in the SE and 1.99 ± 0.09% in the PF group vs. 2.75 ± 0.11% in control group, overall

P< 0.01; P< 0.05, SE and PF groups vs. control group). The percentage of liver weight remained smaller in the SE group (liver: 4.35 ± 0.06% and 4.44 ± 0.08% in control and PF groups respectively vs. 4.21 ± 0.07% in the SE group, P< 0.05; overall P< 0.01).

Plasma leptin concentration was significantly reduced in SE group by over 25% compared with control group (P< 0.05); while pair-feeding led to a small (15 %) reduction, but this did not reach statistical significance (Table 1). Plasma leptin concentration was positively correlated with RpWAT mass within each treatment group (P< 0.05, r = 0.66, 0.82 and 0.64, n = 22, 11 and 23 in the control, PF and SE groups, respectively). Plasma insulin levels were reduced by nearly 40% in the SE group (P< 0.05, compared with control), and was even lower in the PF group (P< 0.05, compared with control, Table 1). However, no statistical difference was observed between the SE and PF groups (P = 0.208). Plasma corticosterone concentration was increased by nearly 40% by cigarette smoke exposure (P< 0.05, compared with control), while it was almost doubled in the PF group (P< 0.05, compared with both control and SE groups, Table 1).

NPY concentration in the AH was increased to a similar level by both smoke exposure and equivalent food restriction (P< 0.05, SE & PF vs. control), while PVN NPY concentration was only significantly increased in the PF group (P = 0.02 PF vs. control). NPY concentration in the Arc was not altered by either SE or equivalent food restriction (Fig. 2). PH NPY concentration was reduced by pair feeding (P = 0.03, PF vs. control), while in the medulla NPY concentration was only increased in the SE group (P< 0.05, SE vs. control). The whole hypothalamic NPY content was significantly increased by smoke exposure (89.3 ± 2.8 ng vs. 75.9 ± 2.4 ng in control, P< 0.01 SE vs. control; overall P< 0.01), with a greater overall increase in the PF mice (100.7 ± 3.4 ng, P< 0.05 compared to both control and SE groups).

As shown in Fig. 3, uncoupling protein (UCP)1 mRNA expression in BAT was significantly decreased in the SE group (P< 0.05 compared to the control group), and the reduction was even greater in the PF group (P< 0.05 compared to both control and SE groups, Fig. 3A). BAT UCP3 mRNA expression in the SE group was increased by more than 60% compared to both control and PF groups (P< 0.05 SE vs. control & PF groups, Fig. 3B). In WAT, tumor necrosis factor (TNF)α mRNA expression was increased by nearly 20% in the PF group (P< 0.05 compared to the control animals), and was more than doubled in the SE group (P< 0.05 SE vs. control & PF groups, Fig. 4A). Interleukin (IL)-6 mRNA expression in WAT was reduced to similar levels by both equivalent food restriction and smoke exposure (P< 0.05, SE & PF vs. control, Fig. 4B). WAT ATGL mRNA expression was only significantly increased in the PF group (P< 0.05 PF vs. control, Fig. 5).

3. Discussion

In the current study mice chronically exposed to cigarette smoke, showed a consistent reduction in chow intake compared with both their pre-exposure baseline, and the non-smoke exposed group. This is consistent with our previous short-term (4 days) and sub-chronic studies (4 weeks) (Chen et al., 2005; 2006). Reduced food intake is commonly observed among human smokers (Albanes et al., 1987), which is also thought to contribute to the pathology of emphysema. However, the decreased food intake caused by smoke exposure is not entirely responsible for the reduced weight gain. Animals with equivalent food restriction (PF animals) gained more weight over the 12 weeks compared with the SE animals.

The similar 30% reduction in WAT mass in PF and SE animals in the face of different body weight suggests the fat loss does not fully contribute to the smaller body weight in the SE

group. In this study, SE mice had reduced liver mass, which may have contributed. About 60% of the free amino acids in the body are thought to derive from muscle (Jagoe and Engelen, 2003). Pronounced lean body mass (mainly skeletal muscle) wasting occurs in smoking related lung diseases, such as chronic obstructive pulmonary disease (COPD) well in advance of when symptoms of muscle wasting becomes apparent (Vestbo et al., 2006). At 12 weeks, mice develop lung inflammation reminiscent of human COPD and show hallmarks of structural changes that, in longer-term studies result in emphysema (unpublished data). Negative energy balance due to inadequate dietary energy intake may exacerbate muscle wasting (Medina et al., 1991; Schols et al., 1991), although it is unlikely that a 15% reduction in intake led to marked changes.

Compared to our earlier observations at 4 weeks where SE mice had greater fat loss (Chen et al., 2006), fat loss in the SE animals was similar to PF animals. In our previous observations at 4 weeks, ATGL was reduced to similar levels in both SE and PF groups. This may suggest that fat deposits are relatively preserved and protein is preferably mobilized during short term food deficiency. However, in the long-term (12 weeks), mild food restriction encouraged fat lipolysis in the face of energy deficiency, as observed in PF animals, and this seemed to be offset by smoke exposure. This is observed in human studies, where long term diet-restriction induced weight loss, fat loss occurred together with reduced lean body mass (Chaston et al., 2006; Layman et al., 2004).

Corticosterone is part of the adaptive physiological response to environmental stressors. The increased plasma corticosterone levels in PF animals may be due to long term food restriction and fat loss, since corticosterone can stimulate feeding and redistribute stored energy into intra-abdominal fat depots (Bergendahl et al., 2000; Bogdan et al., 2001; Dallman et al., 2004;

Fichter and Pirke, 1984). The higher level of corticosterone in the PF versus SE mice suggests that the level of stress imposed by exogenous food restriction (pair feeding) is greater than the voluntary reduction in food intake due to the anorexigenic effect of cigarette smoke exposure. The reduction in plasma insulin concentration in the SE and PF group might be due to the inadequate energy intake and fat loss. However, the level of plasma insulin in the SE group was higher than the PF animals at 12 weeks in the face of similar food intake and fat mass. Further work is required to address whether this affects glucose tolerance.

The Arc is the major hypothalamic site of NPY expression (Morris, 1989). NPY-expressing neurons in the Arc send dense projections to other hypothalamic nuclei important for appetite regulation, including the PVN, dorsomedial hypothalamic nucleus, ventromedial hypothalamic nucleus, and lateral hypothalamus (LH) (Elmquist et al., 1999; Morris, 1989; Schwartz et al., 2000). The hypothalamus also receives NPY inputs that originate from outside the hypothalamus, notably cell groups in the medulla that receive peripheral nutrient signals (Williams et al., 2000). Nicotinic receptors are located in these appetite regulating areas of the hypothalamus (Jo et al., 2002). NPY peptide production is increased by starvation and food restriction, and decreased with refeeding (Beck et al., 1990; Brady et al., 1990; Swart et al., 2002). As a result, NPY concentration was significantly increased by pair feeding in the AH and PVN. Although the orexigenic effect of NPY is mainly exerted in the PVN, NPY terminals are also abundant in the LH and there is a high density of NPY receptors that mediate the orexigenic effects of NPY (Elmquist et al., 1999), regarding as classical "feeding centre". LH was partially included in our AH section. Under normal physiological conditions, hypothalamic NPY gene expression is negatively regulated by leptin signaling (Schwartz et al., 1996; Stephens et al., 1995) and positively by energy deficiency (Kalra et al., 1991; White and Kershaw, 1989). Thus the lower plasma leptin

concentration in the SE group would be expected to stimulate hypothalamic NPY production, which should be even higher than that observed in the PF animals, in line with our previous observations (Furness et al., 2001). However, the increase in PVN NPY was less marked in the SE animals. although the increase in the AH was similar as that in the PF mice, yet with spontaneously reduced caloric intake, suggesting an inhibitory effect of cigarette smoke exposure on both hypothalamic NPY peptide synthesis and its orexigenic function. This anorexigenic effect of moderate smoke exposure is consistent with our previous sub-chronic study, but the changes in hypothalamic NPY is slightly different (Chen et al., 2006). After 4 weeks of SE, PVN NPY was reduced, possibly related to effects of nicotine (Chen et al., 2006; Chen et al., 2007b); whereas here NPY was increased after more chronic exposure to cigarette smoke. We suggest that this may be linked to long term modulation of NPY production via leptin. Our previous and current work showed that chronic (12 weeks) changes in body weight are required to induce significant changes in hypothalamic NPY peptide content (Hansen et al., 2004; Morris et al., 2008).

BAT UCP1 activity is responsible for nonshivering thermogenesis, a major component of thermogenesis in newborn humans and in small mammals. Fasting and chronic food deprivation downregulate UCP1 expression in BAT (Champigny and Ricquier, 1990; Samec et al., 1998; Sivitz et al., 1999). This is well represented in the PF group, where UCP1 expression was markedly reduced to preserve energy expenditure by reducing thermogenesis. The relative preservation of UCP1 mRNA expression in the SE group compared to the PF mice, suggests higher energy expenditure in the SE group compared with the PF mice. This is consistent with previous findings that UCP1 mRNA can be induced by nicotine treatment, probably enhancing energy expenditure (Arai et al., 2001; Yoshida et al., 1999). UCP3 is implicated in the regulation of mitochondrial fatty acid transport and influences basal

metabolic rate (Samec et al., 1998; Schrauwen and Hesselink, 2003; Walder et al., 1998). BAT UCP3 mRNA expression can be downregulated by fasting and food deprivation (Samec et al., 1998; Sivitz et al., 1999). The alternation in UCPs in SE mice may be due to an increase in BAT norepinephrine content stimulated by nicotine, as suggested by a recent paper (Brees et al., 2008; Palou et al., 1998); whereas caloric restriction can reduce norepinephrine turnover in BAT (Young et al., 1982), thus downregulate UCP1 to reserve energy expenditure in a previous study (Valle et al., 2007). An increase in UCP3 would increase the uncoupling of mitochondrial respiration and increase energy expenditure and heat dissipation, further suggesting that smoke exposure encourages energy expenditure. This effect of cigarette smoke exposure on energy homeostasis may crucially contribute to the extra weight loss observed in SE animals compared with the equivalent food restriction of pair feeding. Very little data exist on the effects of nicotine administration on BAT UCP3 mRNA expression in rodents to date, and this experiment more closely models the impact of smoke exposure.

Cigarette smoking alters the production of many pro-inflammatory cytokines from macrophages, such as TNFα and IL-6, which might contribute to the development of lung pathology (Fernandez-Real et al., 2003) and chronic inflammation. The markedly increased WAT TNFα mRNA in the SE group may represent an increase in macrophage infiltration into WAT in response to cigarette smoke exposure, in line with the response in lung. Interestingly no increase in WAT TNFα was observed at 4 weeks (Chen et al., 2006), suggesting a time-dependent effect. IL-6 enhances lipid turnover, stimulating lipolysis as well as fat oxidation. It is not surprising to observe reduced WAT IL-6 expression in both PF and SE groups, where fat mass was reduced. IL-6 can also inhibit the production of TNFα

(Petersen and Pedersen, 2005), which may lead to slightly upregulated TNFα in the PF animals.

The main finding of this study is that chronic, moderate smoke exposure led to a prolonged decrease in energy intake and more pronounced body weight loss than equivalent food restriction, yet with fat loss similar to that observed in the PF mice, possibly in line with data suggesting that cigarette smoke exposure accelerates proteolysis (Mantzoros et al., 1997). Smoke exposure also caused a marked increase in the pro-inflammatory cytokine TNFα after 12 weeks. Total hypothalamic NPY was reduced compared with PF, despite similar reductions in leptin and energy intake, and this may directly contribute to the reduced food intake. However, the higher NPY peptide in SE mice compared with control animals in the face of a voluntarily reduced food intake, suggests an alteration in NPY's orexigenic capacity. Markers of energy expenditure (UCP1 & 3) were elevated by smoke exposure relative to PF, further exaggerating the negative energy balance due to reduced energy intake. Overall these changes may disadvantage COPD patients where weight loss is a strong independent predictor of mortality.

4. Experimental procedure

Balb/C mice were selected based on the inflammatory and cytokine responses in related respiratory studies (Vlahos et al., 2006). Male Balb/C mice (aged 7 weeks, n = 80) were obtained from the Animal Resource Centre Pty. Ltd. (Perth, Australia), and housed at 20 ± 2 °C in micro-isolator cages, and maintained on a 12:12 h light/dark cycle (lights on at 06:00 h). They were allowed a week to adapt to the new environment with *ad libitum* access to standard rodent chow and water. Animals were monitored daily. After acclimatization mice were randomly divided into three groups with similar average body weight, control (sham-

exposed), smoke-exposed (SE), and pair-fed (PF, equivalent food intake as SE animals,) group. The PF group in this protocol allowed us to separate the effect of food restriction induced by smoke exposure from the effects of cigarette smoke exposure per se. The SE animals were exposed to cigarette smoke produced by 1 cigarette (Winfield Red, 16 mg or less of tar, 1.2 mg or less of nicotine and 15 mg or less of CO, Philip Morris, Melbourne, Australia) for 15 min inside a perspex chamber (18 litres, 12 animals per chamber), 3 times a day (09:00, 12:00 and 15:00 h), 5 days a week for 12 weeks. The control and PF groups were handled identically by placing in a chamber of the same size for 15 min but were not exposed to cigarette smoke as previously described (Chen et al., 2006; 2007a). Food intake of the SE group was measured daily and the PF group was given the same amount of chow eaten by the SE group in the previous 24 hours. All mice were weighed twice weekly. The current study was approved by the Animal Experimentation Ethics Committee of the University of Melbourne.

Sample collection

Animals were given *ad libitum* access to food before sacrifice, and all the samples were harvested between 9:00 and 14:00h. Briefly, mice were anaesthetized (ketamine/xylazine 180/32 mg/kg, intraperitoneal), and blood was collected from the abdominal vena cava, and separated plasma was stored at -80°C for subsequent determination of plasma leptin, insulin, and corticosterone concentrations. Mice (n = 12 for the control and PF groups and n = 11 for the SE group) were decapitated and the brain removed and rapidly dissected on ice into regions containing PVN, Arc, anterior and posterior hypothalamus (AH and PH), and medulla as previous described (Chen et al., 2007a). Brain regions were weighed, and stored at -80°C for later determination of NPY peptide content. The rest of the control and SE were harvested differently after bleeding to study lung pathology. Body fat (BAT, RpWAT, and

testicular WAT), liver and kidney were dissected and weighed. BAT and RpWAT were snap frozen in liquid nitrogen and stored at -80°C for later measurement of mRNA expression of UCP1, UCP3, TNFα, IL-6, and ATGL.

Brain NPY, plasma leptin, insulin and corticosterone assays

As previously described (Morris et al., 1986) endogenous NPY from the various brain regions was extracted by boiling in 0.5M acetic acid, and measured by the radioimmunoassay developed in our laboratory using synthetic NPY as standard (10-1280 pg/tube, Auspep, Australia). Plasma leptin, insulin and corticosterone concentrations were measured using commercially available radioimmunoassay kits (Linco, Missouri, USA; MP Biomedicals, Irvine, USA).

UCP1, UCP3, TNFα, IL-6, and ATGL mRNA measurement by real-time PCR

Total RNA was isolated from pooled WAT and BAT using an RNeasy kit (Qiagen, Valencia USA) according to manufacturer instructions. Briefly, the purified total RNA preparation was used as a template to generate first strand cDNA synthesis using SuperScript III (Invitrogen, Carlsbad USA). Quantitative rt-PCR was performed (ABI 7900 HT Sequence Detection System, Applied Biosystem, Foster City USA) using predeveloped primers from Applied Biosystem. Gene expression was quantified by a multiplexing single reaction, where genes of interest (UCP1, UCP3, TNFα, IL-6, and ATGL) were normalized to 18S rRNA. An individual BAT sample from the control group was then arbitrarily assigned as a calibrator against which all other samples are expressed as a fold difference.

Statistical analyses

Results are expressed as mean ± S.E.M. Body weight and 24 hour energy intake of the control, SE and PF mice was analyzed using ANOVA with repeated measures, followed by post hoc LSD tests. Differences in fat and organ mass, plasma leptin, insulin and corticosterone concentrations, brain NPY concentration and content, and expression of mRNA were analyzed using one-way ANOVA, followed by post hoc LSD tests.

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Table 1**:** Effect of 12 weeks of cigarette smoke exposure or PF on food intake, body weight, organ mass and plasma hormones

	Control (n = 22)	**PF (n = 12)**	**SE (n = 23)**	**Overall between groups**
Energy intake (g/24h) (pre-exposure)	2.75 ± 0.17	2.75 ± 0.04	2.77 ± 0.19	
Energy intake (g/24h) (during exposure)	2.71 ± 0.04	2.32 ± 0.03 *	2.31 ± 0.03 *	P< 0.01
Body weight (g, before exposure)	22.0 ± 0.3	21.7 ± 0.4	22.0 ± 0.3	
Body weight (g, end point)	27.6 ± 0.4	25.5 ± 0.5 *	24.6 ± 0.3 *‡	P< 0.01
Liver (mg)	1201.9 ± 20.8	1135.2 ± 36.4	1036.8 ± 23.7 *‡	P< 0.01
Kidney (mg)	190.2 ± 3.4	161.2 ± 3.9 *	170.4 ± 3.4 *	
BAT (mg)	105.0 ± 5.2	112.6 ± 4.3	82.6 ± 1.9 *‡	P< 0.01
RpWAT (mg)	200.3 ± 11.1	125.2 ± 10.6*	137.0 ± 7.0 *	P< 0.01
Testicular WAT (mg)	765.3 ± 39.4	514.3 ± 27.8 *	492.2 ± 27.1 *	P< 0.01
Leptin (ng/ml)	5.93 ± 0.51	5.05 ± 0.41	4.43 ± 0.31 *	P< 0.01
Insulin (ng/ml)	0.55 ± 0.10	0.19 ± 0.03 *	0.34 ± 0.05 *	P< 0.01
Corticosterone (ng/ml)	85.4 ± 7.4	167.7 ± 19.0 *	118.5 ± 11.5 *‡	P< 0.01

Results are expressed as mean ± S.E.M. Data were analysed by one way ANOVA followed by post hoc LSD tests. * Significantly different from control group, P< 0.05. ‡ Significantly different from PF group, P< 0.05.

The n values varied across different parameters, because some control and SE animals were used in other measurements such as lung pathology.

Fig 1: Body weight of the control (open squares, n = 12), PF (astral, n = 12), and SE (filled squares, n = 11) groups during the experimental period. Mice were exposed to cigarette smoke or sham exposed between week 0 and week 12. PF started one day later than smoke exposure. Results are expressed as mean ± S.E.M. Data were analysed by ANOVA with repeated measures followed by post hoc LSD tests.

∗ SE group significantly different from the control group, $P < 0.05$

† PF group significantly different from the control group, $P < 0.05$

‡ SE group significantly different from the PF group, $P < 0.05$

Fig 2: NPY-LI (expressed as ng/mg tissue) in the brain regions of the control (open bars, n = 12), PF (stippled bars, n = 12) and SE (filled bars, n = 11) groups at 12 weeks. Areas shown are anterior hypothalamus (AH), paraventricular nucleus (PVN), arcuate nucleus (Arc), posterior hypothalamus (AH), and medulla (Med). Results are expressed as mean ± S.E.M. Data were analysed by one way ANOVA followed by post hoc LSD tests. Overall P value among 3 groups: AH $P < 0.01$, PVN $P = 0.02$, Arc $P < 0.01$, PH $P < 0.01$, medulla $P < 0.01$.

∗ Significantly different from the control mice, $P < 0.05$

Fig 3: UCP1 (A) and UCP3 (B) mRNA expression in BAT in the control (open bars), PF (stippled bars), and SE (filled bars) groups at 12 weeks. Results are expressed as a fold difference relative to the value for control BAT for pooled samples. Data were analysed by one way ANOVA followed by post hoc LSD tests. Overall $P < 0.01$ for UCP1 and UCP3 among 3 groups.

* Significantly different from the control group, $P < 0.05$

‡ Significantly different from the PF group, $P < 0.05$

Fig 4: TNFα (A) and IL-6 (B) mRNA expression in WAT in the control (open bars), PF (stippled bars), and SE (filled bars) groups at 12 weeks. Results are expressed as a fold difference relative to the value for control BAT for pooled samples. Data were analysed by one way ANOVA followed by post hoc LSD tests. Overall P value among 3 groups: TNFα $P< 0.05$, IL-6 $P < 0.01$.

∗ Significantly different from the control group, $P< 0.05$

‡ Significantly different from the PF group, $P< 0.05$

Fig 5: ATGL mRNA expression in WAT in the control (open bars), PF (stippled bars), and SE (filled bars) groups at 12 weeks. Results are expressed as a fold difference relative to the value for control BAT for pooled samples. Data were analysed by one way ANOVA followed by post hoc LSD tests. Overall $P = 0.03$ among 3 groups.

∗ Significantly different from the control group, $P< 0.05$

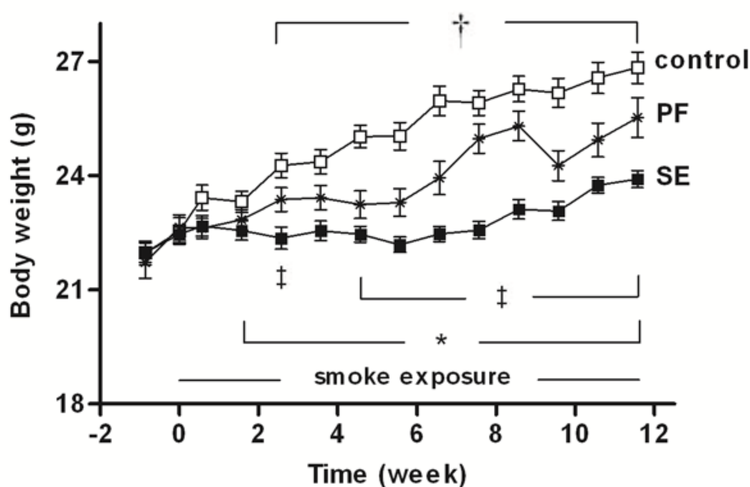


Figure 1

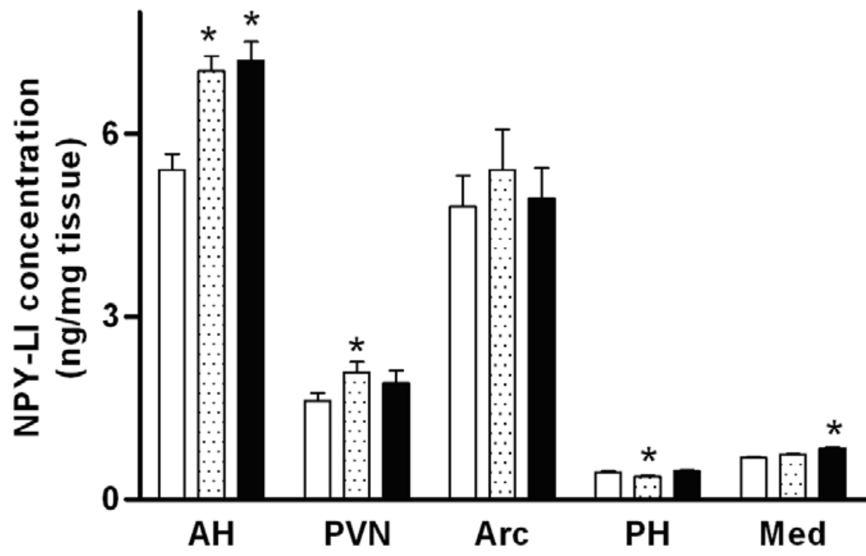


Figure 2

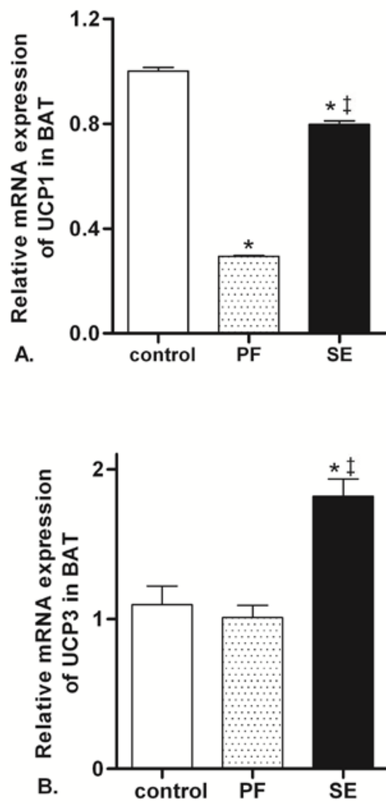
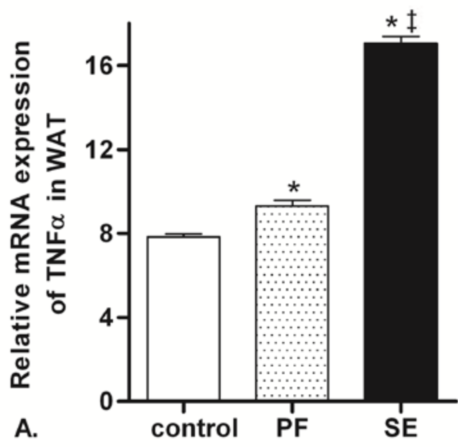


Figure 3



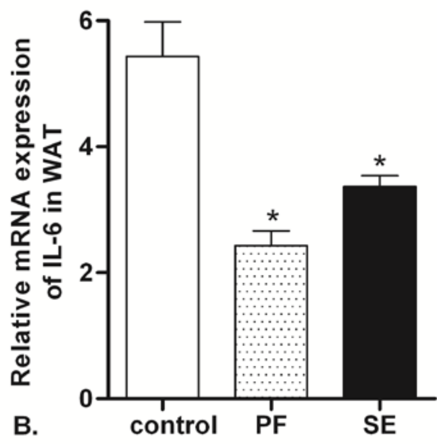


Figure 4

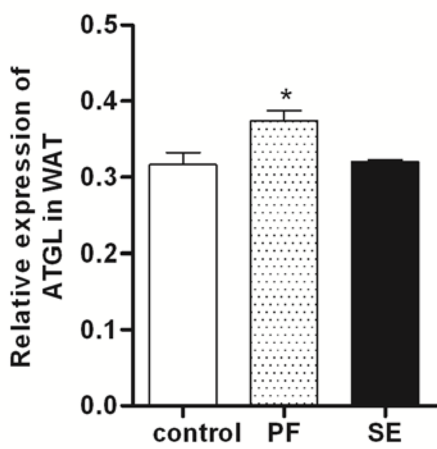


Figure 5